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(54) Title: NUCLEOZYMES

(57) Abstract

Nucleozymes containing ribonucleotides and deoxyribonucleotides or nucleic acid analogues are described herein. The nucleozymes have catalytic activity and are significantly more resistant to degradation than their all-RNA ribozyme connterparts. Also described are methods for preparing the nucleozymes along with methods of using nucleozymes, e.g., as therapeutic agents.

CORRECTED VERSION *

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(54) Title: MODIFIED RIBOZYMES

(57) Abstract

The present invention refers to an RNA molecule with catalytic activity comprising at least one modified nucleoside, wherein the hydroxy group at the 2'-position of the ribose sugar is replaced by a modifier group, selected from halo, sulfhydryl, azido, amino, monosubstituted amino and disubstituted amino groups, a process for the preparation of modified RNA molecules and the use of modified RNA molecules as therapeutic agents and biocatalysts.

Modified Ribozymes

SPECIFICATION

Certain naturally occuring ribonucleic acids (RNAs) are subject to self-cleavage. The first reported example is the cleavage of the ribosomal RNA precursor of the protozoan Tetrahymena (for a review see Cech, Ann.Rev.Biochem. 59 (1990), 543-568) which requires guanosine as cofactor. A number of examples of RNA cleavage have been subsequently discovered in viroid, virusoid and satellite RNAs (for reviews see Sheldon et al. in Nucleic Acids and Molecular Biology (1990) Vol. 4, pg. 227-242, ed. F. Eckstein and D.M.J. Lilley, Springer Verlag Berlin Heidelberg; Symons, TIBS 14 (1989), 445-450). These cleavages involve site-specific breakage of a phosphodiester bond in the presence of a divalent cation such as Mg^{2+}, generating a 5'-hydroxyl and a 2',3',-cyclic phosphodiester terminus. Sequence analysis around the site of self-cleavage of several of such RNAs has led to the identification of a common structural feature essential for cleavage which was named a "hammerhead" structure (Hutchins et al., Nucleic Acids Res. 14 (1986) 3627-3640). This structure consists of three helices and 13 conserved nucleotides (framed in below scheme) which form a three dimensional structure amenable to cleavage at one particular position. The self-catalyzed cleavage is normally an intramolecular process, i.e. a single RNA molecule contains all the functions necessary for cleavage. However, Uhlenbeck (Nature 328 (1987), 596-600) has demonstrated that this hammerhead structure does not have to be embodied in one strand but can be made up of two strands. These two strands combine to form the hammerhead structure which leads to phosphodiester bond cleavage (indicated by an arrow) in one of the strands (strand S) whereas the other (strand E) remains unaltered and can participate in many cleavage reactions. This strand meets the definitions of an enzyme and is called a ribozyme. Whereas the framed sequences (below scheme) are conserved the others may vary provided that the

viral genes and particular ndogenous genes. This requires the construction of a RNA molecule which is able to form a hammerhead or a hairpin structure with the target RNA and to cleave this at a predetermined position. A first application to the inhibition of the HIV-1 virus by this strategy has been reported (Sarver et al., Science 247 (1990), 1222-1224). Other examples of the action of targeted hammerhead ribozymes in vivo are Cammeron and Jennings (Proc.Natl.Acad.Sci. USA 86 (1986), 9139-9143) and in vitro Cotten et al. (Mol.Cell.Biol. 9 (1989), 4479-4487).

Further, other useful catalytic properties of ribozymes are known, e.g. dephosphorylase and nucleotidyl transferase activities (see Patent Application WO88/04300). Therein RNA enzymes are disclosed which are capable of dephosphorylating oligonucleotide substrates with high sequence specifity, which distinguishes them from known protein enzymes. RNA molecules also can act as RNA polymerases, differing from protein enzymes in that they use an internal rather than an external emplate. Thus, various heteropolymers can be constructed by variant RNA enzyme forms. This enables the formation for example of messenger RNA molecules for particular proteins or peptides. Furthermore, Herschlag and Cech (Nature 344, (1990), 405-409) describe an RNA enzyme with DNase acitivity.

To be useful as a therapeutic agent the RNA enzyme has to be introduced into target cells. There are a priori two methods for delivery of the ribozyme into the target cells:

(a) exogenous delivery of a preformed synthetic RNA;
(b) endogenous transcription of a ribozyme-coding gene located on a plasmid.

A great disadvantage of method (a) resides in the very low stability of RNA molecules under physiological conditions due to their fast degradation by a variety of ribonuclease

activity comprises a sequence specific endoribonuclease activity. More preferably the RNA is a hammerhead ribozyme as described above. Especially preferred is that the ribozyme can combine with another RNA strand to form a hammerhead structure consisting of two strands, wherein the modified RNA strand is the E strand as described above.

Although a hammerhead ribozyme is especially preferred, other RNA enzymes are encompassed also by the present invention, e.g. the Tetrahymena ribozyme (Cech, Ann.Rev.Biochem. 59 (1990), 543-568) in naturally occuring form or a shortened form thereof (Zang et al., Biochemistry 27 (1988), 8924-8931), and especially the Hairpin RNA (Hampel et al., Nucleic Acids Res. 18 (1990) 299-304) or RNA containing proteins such as the RNase P (Baer et al., in Nucleic Acids & Molecular Biology (1988), Vol. 3, pp 231-250, ed. F. Eckstein and D.M.J. Lilley, Springer Verlag Heidelberg), the telomerase (Greider and Blackburn, Nature 337 (1989), 331-337).

The incorporation of a modifier group at the 2'-position of the ribose sugar appears also to be particularly useful for RNA with new functions either derived at by a procedure that depends on alternate cycles of selection (Tuerk and Gold, Science 249 (1990), 505-510; Ellington and Szostak, Nature 346 (1990), 818-822) or any other method .

The modifier group replacing the hydroxy group at the 2'-position of the ribose sugar is selected from halo, sulfhydryl, azido, amino, monosubstituted amino, and disubstituted amino groups. The halo group can be a fluoro, chloro, bromo or iodo group, wherein the fluoro group is preferred. The substituents of the substituted amino group are preferably C_1-C_3 alkyl and or hydroxyalkyl groups. Most preferably the modifier group is a halo or an unsubstituted amino group.

5-position with halo or C_1-C_5 alkyl groups, especially bromo or methyl groups. Most preferably the nucleotide base attached to the modified ribose sugar is uracil.

The modified nucleosides which are incorporated into a RNA molecule are either previously described compounds or compounds which can be prepared in analogy to known compounds. The mostly preferred fluoro and amino analogs of ribonucleosides have been described previously, 2'-deoxy-2'-fluorocytidine (Doerr & Fox, J.Org.Chem. 32 (1967), 1462; Mengel & Guschlbauer, Ang.Chem. 90 (1978), 557-558); 2'-deoxy-2'-fluoroadenosine (Ikehara & Miki, Chem.Pharm.Bull. 26 (1978), 2449-2453), 2'-deoxy-2'-fluorouridine (Codington et al., J.Org.Chem. 29 (1964), 558-564), 2'-deoxy-2'-aminouridine (Verheyden et al., J.Org.Chem. 36 (1971), 250) and 2'-deoxy-2-aminocytidine (Verheyden et al. (1971) supra). For the synthesis of some of these compounds more recent synthetic procedures can be employed. The 2'-deoxy-2'-fluorocytidine can be prepared from 2'-deoxy-2'-fluorouridine by the method of Sung (J.Org.Chem. 47 (1982), 3623-3628). The same method can be used for the transformation of 2'-deoxy-2'-azidouridine to 2'-deoxy-2'-azidocytidine (Verheyden et al. (1971), supra). The latter can be reduced to 2'-deoxy-2'-aminocytidine by the method of Mungall et al. (J.Org.Chem. 40 (1975), 1659).

The synthesis of the 2'-deoxy-2'-fluoronucleoside 5'-triphosphates can be carried out either according to Ludwig (Acta Biochim. et Biophys. Acad.Sci.Hung. 16 (1981), 131-133) or Ludwig and Eckstein (J.Org.Chem. 54 (1989), 631-635). The 2'-deoxy-2'-aminouridine and -cytidine 5'-triphosphates can be prepared as described for the diphosphates by Hobbs et al. (Biochemistry 12 (1973), 5138-5145) with the modification that pyrophosphate is employed instead of phosphate. The 2'-deoxy-2'-fluoronucleoside 3'-phosphoramidites for automated oligonucleotide synthesis can be prepared by the method of

nucleosides are modified and which are unmodified) which is d signated as a so-called "selectiv modification pattern". An RNA comprising selective modification pattern is a molecule wherein nucleosides at specifically selected locations can be modified while nucleosides at other specifically selected locations can be unmodified. For instance, nucleotides which are known to be hypersensitive sites for ribonucleases (e.g. due to the secondary structure of the RNA molecule) should be modified to achieve an extended life time of the RNA molecule. An example for a ribonuclease-hypersensitive site is provided at position 21 of ribozyme E1. As shown in Fig. 3 the RNA molecule is cleaved at this position by RNase A with very high intensity.

Still a further embodiment of the present invention is a RNA molecule additionally comprising at least one modified internucleotidic phosphodiester linkage. Examples for suitable modified phosphodiester linkages are methyl phosphonate groups or phosphorothioate groups, the latter being especially preferred. Preferably at least the 5'-terminal phosphodiester linkage and/or the 3'-terminal phosphodiester linkage of the RNA molecule is modified. More preferably the 5'-terminal phosphodiester linkage and the last three 3'-terminal phosphodiester linkages are modified.

It was found, that the presence of modified internucleotidic linkages alone was not sufficient to provide increased stability against degradation. However, the combined presence of 2'-modified ribose sugars together with modified internucleotidic linkages showed an additive stability enhancing effect. A more than fiftyfold increase in stability confered by both modifications outweighs the decreased efficiency in cleavage compared to a unmodified ribozyme.

precursors. After the coupling of the precursor to the RNA chain has taken place the subsequent oxidation step, however, is not performed with iodine, as in the case of non-modified linkages, but with sulfur or a sulfurating agent, whereby the phosphorothioate group is obtained.

The chemical synthesis of modified RNA molecules is carried out in analogy to that of unmodified RNA or DNA molecules, which is known in the art. More specifically the RNA synthesis is carried out by chemical synthesis on solid support involving the stepwise addition of the respective nucleotide precursors. After having synthesized an RNA product of the desired length, the RNA is removed from the solid support by conventional means and purified, preferably by gel electrophoresis. Alternatively the chemical RNA synthesis can also be carried out by any other known technique without using a solid support. E.g. the RNA can be synthesized in a soluble form and subsequently purified by means known in the art.

When the 2'-amino modifier group is incorporated into the RNA chain it has to be protected before the phosphitylation reaction (i.e. the preparation of the nucleotide precursor) and for subsequent use in the coupling reactions. For this purpose the trifluoroacetyl group is preferably used as a protecting group, because it is stable during the cycles of synthesis on the nucleic acid synthesizer and is removable under the conventional treatment with ammonia.

Alternatively the synthesis of the RNA chain can be carried out by transcription from a nucleic acid template by an appropriate nucleic acid polymerase. Preferably the template is a DNA template and the nucleic acid polymerase is a DNA dependent RNA polymerase. Mor preferably the DNA dependent RNA polymerase is selected from the group consisting of T7, T3 and SP6 polymerases, which are highly processive

rang of Km variation observed for hammerhead systems with different base composition (Fedor & Uhlenbeck, supra). In addition, very surprisingly the incorporation of a single 2'-aminouridine immediately 5' of the site of cleavage in the substrate increased the kcat markedly (table 1), so that it is conceivable to produce ribozymes of enhanced activity by the selective introduction of 2'-modified nucleosides at specific sites. These results definitely show that there is no requirement for the presence of 2-hydroxyl groups throughout the enzyme part of the hammerhead structure for catalytic activity but that the modifications according to the present invention are tolerated at least in certain positions. In contrast, the incorporation of only 15 % 2'-deoxynucleotides into a hammerhead ribozyme is reported to decrease the catalytic efficiency by two orders of magnitude, while not affecting the K_m (Perreault et al. (1990), supra). Since the rate of cleavage is determined by the angle of attack of the 2'-hydroxyl on the phosphorus at the site of cleavage, it is greatly influenced by the overall structure of the hammerhead system. Thus, the observed influence of 2'-modifications on the rate supports the notion that the 2'-fluoro analogs adopt a structure more similar to that of ribonucleotides than that of deoxyribonucleotides. This apparently also holds for the amino analogs. The other 2'-modified nucleosides according to the present invention exhibit similar catalytic activity.

A still further object of the present invention is the use of RNA molecules with catalytic activity comprising at least one modified nucleotide, as therapeutic agents, especially for the specific cleavage of viral or other foreign genetic material or transcripts from viral or other foreign genetic material, or as biocatalyst in biochemical or biotechnological processes. For these purposes the RNA molecules of the present invention seem to be more suitable than their unmodified analogs, because of their increased stability against chemical and/or enzymatical cleavage.

EXAMPLES

Example 1

Preparation of oligoribonucleotides

Automated synthesis of oligoribonucleotides: Automated oligoribonucleotide synthesis was carried out with an Applied Biosystems 380B DNA Synthesizer on a 1 μmol scale using the monomeric ribonucleotide phosphoramidites supplied by Milligen/Biosearch. Control pore glass columns with the ribonucleoside coupled to it were either from Milligen/Biosearch or Peninsula. The oligomers were worked up according to the specifications of the supplier of the ribonucleotide phosphoramidites (Milligen/Biosarch). After removal of the protecting groups the oligoribonucleotides were concentrated by spin dialysis on Amicon filter membranes centricon 10 and ethanol precipitated. The dried pellets were taken up in 50 μl water and subjected to PAGE. Bands were visualized by UV shadowing, cut out and the RNA was isolated by eluting at 37°C overnight in buffer (0.25 M ammonium acetate, 10 mM TRIS/HCl pH 8.0, 1 mM EDTA) (Fedor & Uhlenbeck, PNAS USA 87 (1990), 1668-1672). Concentrations were determined using the extinction coefficient per nucleotide of 6600 $M^{-1}cm^{-1}$ given in the literature (Fedor & Uhlenbeck 1990). Aqueous solutions of the oligoribonucleotides were stored at -20°C.

Construction of plasmids containing templates for run off transcription:

The following oligodeoxynucleotides were synthesized for the plasmid construction by the phosphoramidite method with an Applied Biosystems 380B DNA synthesizer:

RS2-T,5'-d(GATATCCTGACTCCCTATAGTGAGTCGTATTA)-3; RS2-C,5'-d(TAATACGACTCACTATAGGGAGTCAGGATATCTGCA)-3'; RE1-T,5'-d(GGAGTTTCGGCCTAACGGCCTCATCAGAGGACCCTATAGTGAGTCGTATTA)-3' and RE2-C,5'-d(TAATACGACTCACTATAGGGTCCTCTGATGAGGCCGTTAGGCCGAAACTCCTGCA)-3'.

Figure 1 shows autoradiographs of T7 RNA polymerase run ff transcriptions of pUCRS after PAGE. A: The transcription was performed on a 150 µL scale in the presence of 20 mM $MgCl_2$ and 2.5 mM each of the four nucleoside triphosphates at 37°C for 3 h. The reaction mixture was dephosphorylated with alkaline phosphatase and 5'-^{32}P-labeled by reaction with polynucleotide kinase and [γ-^{32}P]-ATP. The labeled transcription mixture was subjected to PAGE. B: The transcription was performed on a 150 µL scale at 37°C for 3 h in the presence of 20 mM $MnCl_2$, 0.5 mM ATP, 25 µCi [α-^{32}P]-ATP, 2.5 mM CTP and GTP, and 2.5 mM 2'-fluorouridine triphosphate. The transcription mixture was directly subjected to PAGE. The asterisks mark ^{32}P-labeled phosphates. 'N' denotes any nucleotide added by T7 RNA polymerase beyond the full length of the template DNA (c.f. Milligan and Uhlenbeck, Meth.in Enzymology 180A (1989), 51-62).

Figure 2 shows an autoradiograph of T7 RNA polymerase run off transcripts of pUCRE 16 containing 2'-aminouridine after PAGE. Lane 1: 2'-aminouridine containing 34-mer marker E3, synthesized chemically. Lane 2: The transcription was performed on a 150 µl scale at 37°C for 3 h in the presence of 20 mM $MgCl_2$, 60 µCi [α-^{32}P]ATP, 1 mM CTP and GTP, and 1 mM 2'-aminouridine triphosphate. The transcription mixture was directly applied PAGE.

Preparation of oligoribonucleotides: The following oligoribonucleotides were prepared
a.) by run off transcription (sequences given without the 5'-triphosphate):
E1, 5'-GGGUCCUCUGAUGAGGCCGUUAGGCCGAAACUCC-3';
E2,,5'-GGG(2'-FU)CC(2'-FU)C(2'-FU)GA(2'-FU)GAGGCCG(2'-FU)(2'-FU)AGGCCGAAAC(2'-FU)CC-3' and
E3,5'-GGG(2'-NH_2U)CC(2'-NH_2U)C(2'-NH_2U)GA(2'-NH_2U)GAGGCCG(2'-NH_2U)(2'-NH_2U)AGGCCGAAAC(2'-NH_2U)CC-3';

Total degradation of oligoribonucleotides by RNAse A: The oligoribonucleotides S1 and S2 were digested with RNase A after 5'-^{32}P labeling according to the following protocol: The oligomer (8.5 µM in a final volume of 20 µl) was reacted with 1.25 x 10^{-3} Units of RNAse A in buffer containing 50 mM TRIS/HCl pH 7.5 and 10 mM $MgCl_2$ for 10 min at 37°C. Products were analyzed by PAGE.

Figure 3 shows an autoradiograph of partial Ribonuclease A cleavage of 5'-labeled run off transcripts E1 and E2 separated by PAGE. Conditions as described before. The numbered lanes correspond to 1) no enzyme added, 2) $2x10^{-4}$ units RNase A, 3) $3x10^{-5}$ units RNase A, 4) $8x10^{-6}$ units RNase A, 5) $16x10^{-7}$ units RNase A. Base numbering was facilitated by counting the bands of a Mn^{2+} mediated cleavage of the unmodified transcript (10 µmoles RNA heated to 90°C for 3 min in 10 mM $MnCl_2$). The circled numbers indicate the bands expected from RNase-A susceptible cleavage positions. Arrows indicate the bands that arise from cleavage 3' to uridine and which are absent in the lanes where 2'-fluorouridine containing ribozyme was cleaved.

Figure 4 shows an autoradiograph of the total degradation of S1 and S2 by RNase A after PAGE. Details of the reaction are as described above. Lane 1: total digestion of 12-mer S2; lane 2: total digestion of 12-mer S1; lane 3: cleavage ladder of the 34-mer E1 by reaction with 20 mM $MnCl_2$ at 90°C for 3 min as a length standard. The product of cleavage of S2 is 1 nucleotide longer than that of S1 indicating the presence of 2'-aminouridine at position 6.

However, potentially more interesting for future applications is the question whether the presence of this modification in the enzyme part of the ribozyme will interfere with its catalytic activity. Thus, the reaction of the 2'-fluorouridine-containing ribozyme E2 with the unmodified substrate S1 was investigated. Indeed, the gel analysis indicated that the substrate was cleaved with similar efficiency as the pair E1 and S1. The catalytic constants of the 2'-fluorouridine-containing ribozyme E2 were determined (Fig. 6) and compared to those of the unmodified ribozyme E1. This comparison reveals that the second order rate constant for the former (k_{cat}/K_m = 0.0026 nM^{-1}) is one order of magnitude smaller than that of the latter (k_{cat}/K_m = 0.023 nM^{-1}) (Fedor & Uhlenbeck (1990), supra) (Table 1). This decrease in catalytic efficiency is primarily due to a decrease in the rate of cleavage, whereas the Km values for both ribozymes is nearly identical. This reduced rate of cleavage, however, lies well within the range of cleavage efficiencies observed for various hammerhead systems with different base compositions (Fedor & Uhlenbeck (1990), supra). Hammerhead ribozyme reactions can be carried out with $MgCl_2$ as well as with $MnCl_2$ as metal ion cofactor, where the half life of cleavage is decreased in the presence of the latter cofactor by about 10 fold (Uhlenbeck, Nature 328 (1987), 596-609). Such a decrease in the half life of the substrate under cleavage conditions upon switching from Mg^{2+} to Mn^{2+} was also observed for the reaction of 2'-fluorouridine-containing enzyme E2 with substrate S1. Thus the metal ion requirement for the cleavage reaction is not altered by the incorporation of 2'-fluoronucleotide analogs.

The effect of the presence of 2'-aminouridine in the ribozyme was also investigated. When the 2'-aminouridine containing ribozyme E3 is reacted with nonmodified substrate S1, the catalytic efficiency drops an order of magnitude to k_{cat}/K_m = 0.0015 nM^{-1}. This decrease in efficiency is clearly due to a

range of variations observed for hammerhead systems of different bas composition. It also becomes evident that it is possible to increase the catalytic efficiency by selectively introducing 2'-modifications at specific positions. While the latter effect was demonstrated for the substrate oligoribonucleotide, it is anticipated that a similar influence on catalysis can be found for selective modifications in the enzyme.

Figure 5 shows an autoradiograph of the cleavage of 2'-fluorouridine and ^{32}P-AMP-containing substrate S3 by ribozyme E1. The cleavage reaction was performed in the presence of 10 mM $MgCl_2$ in 50 mM TRIS/HCl, pH 7.5 on a 40 µl scale at 25°C. The concentration of E1 and S3 was 2.5 µM and 7.5 µM, respectively. All other details are as described above (c.f. Determination of Cleavage Kinetics). At the indicated times 10 µl aliquots were transferred into 10 µl water and 10 µl urea stop mix prior to PAGE. Lane 1: reaction after 0.5 min; lane 2: reaction after 15 min; lane 3: reaction after 30 min. The asterisks mark ^{32}P-labeled phosphates.

Figure 6 shows an Eadie-Hofstee plot of the ribozyme reaction of E2 with S1. The cleavage reaction were performed on a 20 µl scale in the presence of 10 mM $MgCl_2$ with a 10 nM concentration of E2 and concentrations of S1 of 50 nM, 100 nM, 200 nM, 400 nM, 500 nM, and 700 nM. After 7 min 10 µl aliquots were transferred into 10 µl water and 10 µl urea stop mix prior to PAGE. It was established previously that these time points fall within the linear range of initial velocities. The autoradiographs were evaluated by integration of their optical density on a laser densitometer.

Figure 7 shows an Lineweaver-Burk plot of the ribozyme reaction of E3 with S1. The cleavage reactions were performed on a 20 µl scale in the presence of 10 mM $MgCl_2$ with a 10 nM

catalyzed cleavage. Hammerhead ribozymes targeted against this site were chemically synthesized. The nucleotide sequence of the unmodified hammerhead enzyme RE115 is given in Fig. 9.

Cleavage Kinetics with LTR RNA: k_{cat}/K_m values were determined under single turnover conditions. Ribozymes were preincubated at 75°C for 1 min in the presence of 50 mM Tris-Cl pH 7.5 followed by 5 min of incubation at 37°C. $MgCl_2$ was added to a final concentration of 10 mM and the solutions were again incubated for 5 min at 37°C. LTR RNA was directly used as an aqueous solution. The reaction mixture (10 µl) contained between 20 nM and 1 µM ribozyme, 50 mM Tris-Cl pH 7.5 and 10 mM $MgCl_2$. The reaction was started by addition of LTR RNA to a final concentration of 10 nM. After 1 hour at 37°C the reaction was stopped by addition of 10 µl stop mix and analysed by 4 % PAGE (40 cm long, 8 M urea). After 1 h electrophoresis at 50 W followed by autoradiography the fraction of noncleaved LTR RNA was determined by laser scanning densitometry. k_{cat}/K_m values were obtained by plotting the remaining fraction of LTR RNA (Frac S) against the ribozyme concentration ([RE]) according to the following equation:

$$k = \frac{\ln(FracS)}{t} = [RE]\frac{k_{cat}}{K_m},$$

where k is the observed reaction rate and t is the reaction time of 1 h.

In order to investigate the influence of chemical modifications on the catalytic efficiency of a ribozyme several analogs of RE115 containing 2'-fluoro or 2'-deoxy substitutions and/or terminal phosphorothioate linkages were synthesized. Whereas 2'-fluorocytidine substitutions hat no

Example 5

Stability of oligoribonucleotides

The ribozymes of Example 4 were examined for their stability against nuclease digestion.

Test conditions:

Molt 4 clone 8 cells (kindly supplied by E. Jurkiewicz, Deutsches Primatenzentrum, Göttingen) grown in medium RMPI 1640 to a cell density of about 10^6 cells/ml were centrifuged at 1000 g for 5 min in a Heraeus Minifuge. 5'-^{32}P-labeled ribozymes were pre-heated for 1 min at 90°C, chilled on ice, added to the cell supernatant to a final concentration of 300 nM and incubated at 37°C. Aliquots were taken at the indicated time points and analysed by 20 % PAGE containing 8 M urea followed by autoradiography.

Results:

More than 80 % of RE115 was degraded after 2 min incubation in the cell supernatant as indicated by denaturing PAGE. For RE115(S) similar results were obtained. However, no degradation of RE115(FC,FU,S) within 1 hour was observed. A comparison with the rate of degradation of the unmodified ribozyme indicates that the combination of 2'-modified pyrimidine nucleosides and terminal phosphorothioate linkages results in an estimated increase of more than fiftyfold of ribozyme stability against digestion by nucleases present in T cell supernatant. 2'-modified ribozymes without phosphorothiate group show a stability which is about two times lower than the stability of RE115 (FC,FU,S).

8. RNA according to claim 7, wherein the substituted nucleotide base is selected from the group consisting of xanthine, hypoxanthine, 2,6-diamino purine, 2-hydroxy-6-mercaptopurine and purine bases substituted at the 6-position with sulfur or pyrimidine bases substituted at the 5-position with halo or C_1-C_5 alkyl groups.

9. RNA according to claim 7, wherein the nucleotide base attached to the modified ribose sugar is a base naturally occuring in RNA.

10. RNA according to claim 9, wherein the nucleotide base attached to the modified ribose sugar is a pyrimidine base.

11. RNA according to any of the preceding claims, wherein all nucleotide bases of one specific kind are attached to a modified ribose sugar.

12. RNA according to claim 11, wherein all uracil nucleotide bases are attached to a modified ribose sugar.

13. RNA according to claim 11, wherein all cytosine nucleotide bases are attached to a modified ribose sugar.

14. RNA according to any one of the claims 1 - 10, wherein all nucleotide bases of two specific kinds are attached to a modified ribose sugar.

15. RNA according to claim 14, wherein all cytosine and uracil nucleotide bases are attached to a modified sugar.

24. RNA according to any of the claims 20 - 23, wherein the 5'-terminal phosphodiester linkage and the last three 3'-terminal phosphodiester linkages are modified.

25. Process for the synthesis of an RNA molecule with catalytic activity, comprising:
incorporating into an RNA chain at least one modified nucleotide, wherein the hydroxy group at the 2'-position of the ribose sugar is replaced by a modifier group, selected from halo, sulfhydryl, azido, amino, monosubstituted amino and disubstituted amino groups.

26. Process according to claim 25, wherein the modifier group is a halo or an amino group.

27. Process according to claim 25 or 26, wherein the halo group is a fluoro group.

28. Process according to any of the claims 25-27, wherein the synthesis of the RNA chain is carried out by chemical synthesis from nucleotide precursors on solid support, removing the RNA product from said solid support and purifying the removed RNA product.

29. Process according to any of the claims 25-27, wherein the synthesis of the RNA chain is carried out by chemical synthesis from nucleotide precursors in solution and purifying the RNA product.

30. Process according to claim 28 or 29, wherein the respective phosphoramidites or H-phosphonates are used as nucleotide precursors.

39. Process according to any of the claims 34 - 37, wherein the modifier group is a amino, monosubstituted amino, or disubstituted amino group and the synthesis of the RNA chain is carried out in presence of Mg^{2+} ions.

40. Use of an RNA according to any of the claims 1 - 24 as a therapeutic agent or a biocatalyst.

41. Therapeutic agent comprising as active ingredient an RNA according to any of the claims 1 - 24, optionally together with convenient fillers, adjuvants, carriers and diluents.

42. Process of preparing a therapeutic agent, wherein the therapeutic agent comprises as active ingredient an RNA according to any of the claims 1 - 24, optionally together with convenient fillers, adjuvants, carriers and diluents.

Dated this 2nd day of March, 1994

Max-Planck-Gesellschaft zur Forderung der Wissenschaften e.V.

By its Patent Attorneys
Davies Collison Cave

Fig. 2

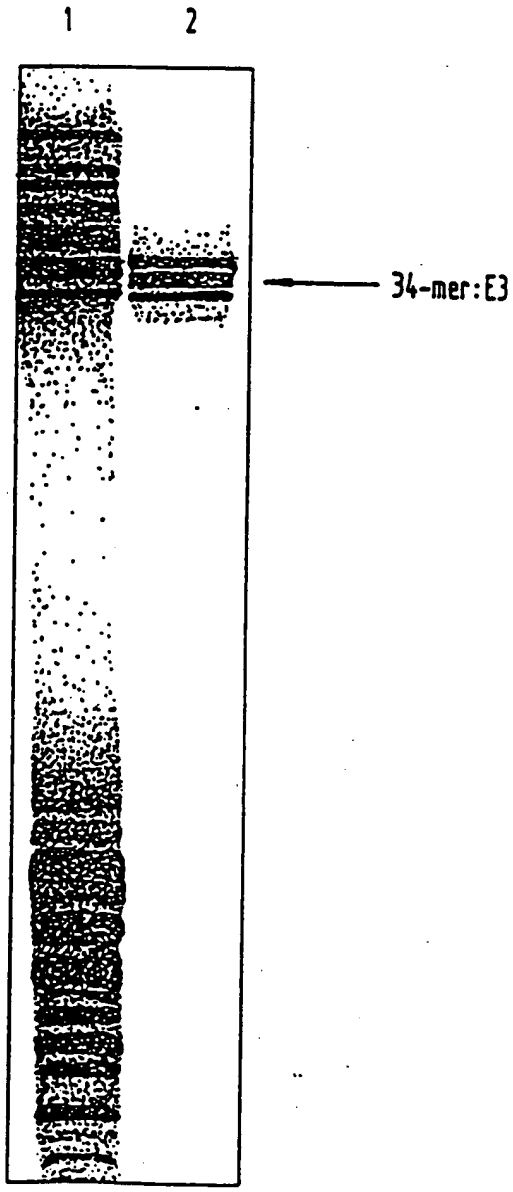


Fig. 4

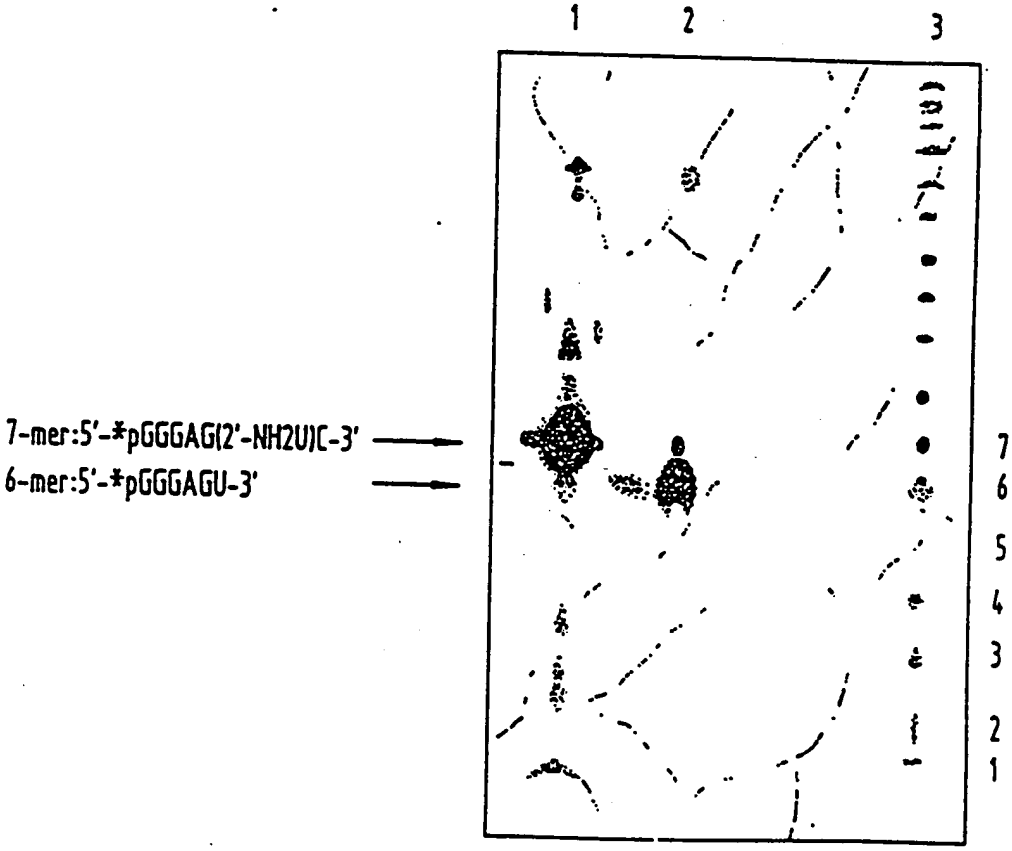


Fig.6

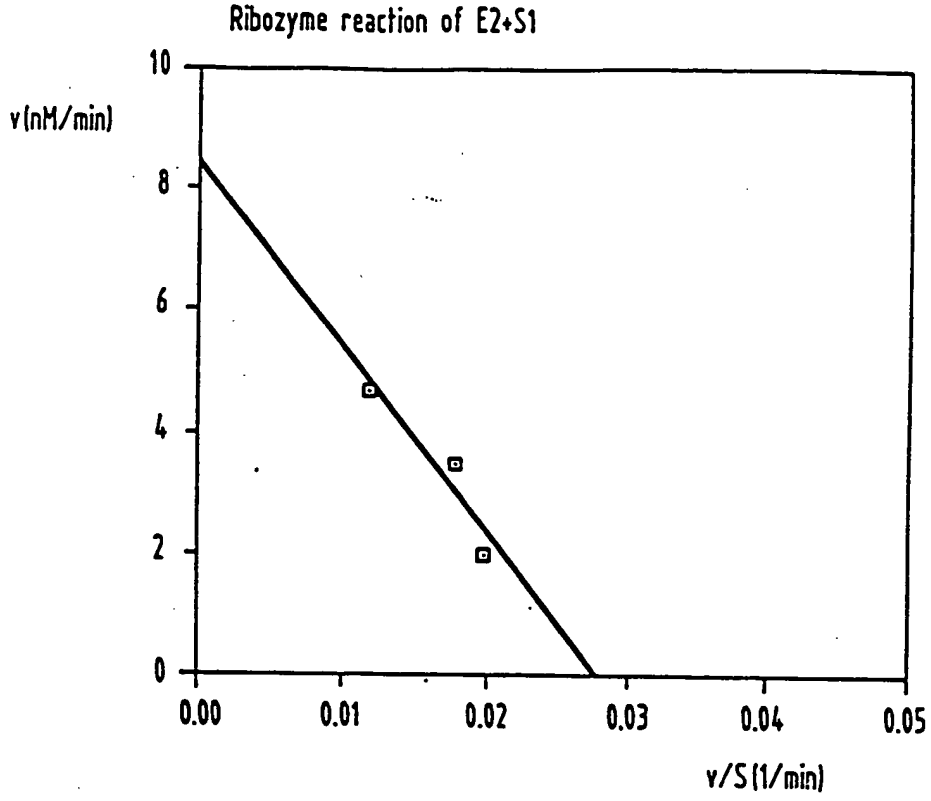
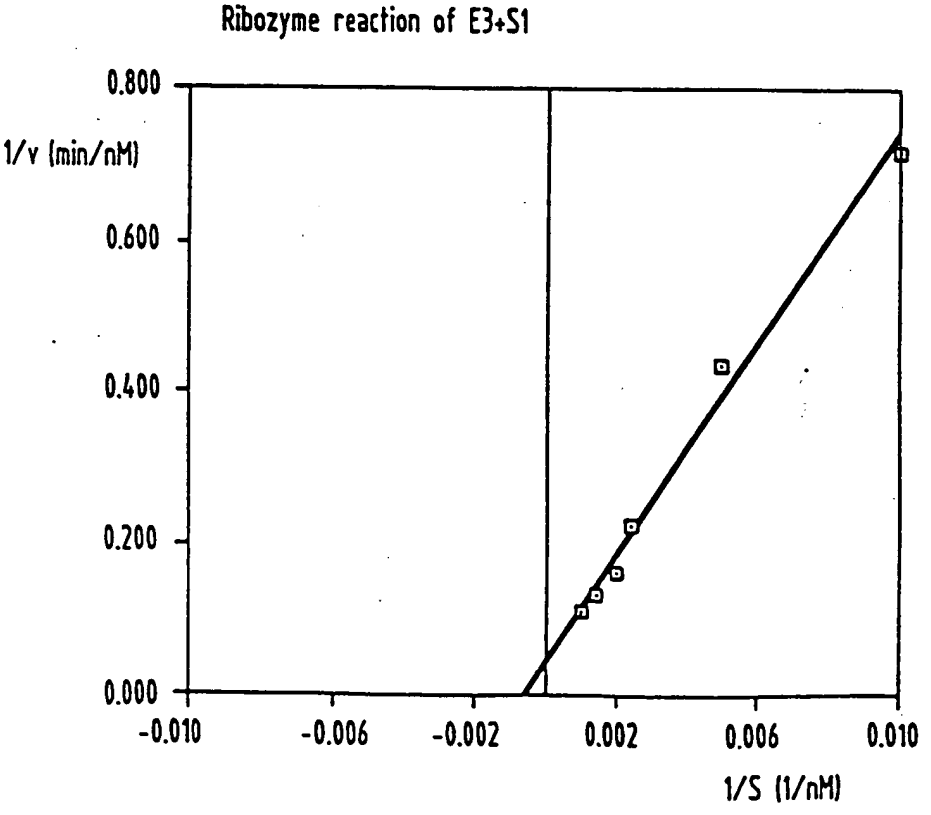

Ribozyme reaction of E2+S1
v(nM/min)
10
8
6
4
2
0
0.00
0.01
0.02
0.03
0.04
0.05
v/S (1/min)

Fig.7


Ribozyme reaction of E3+S1
0.800
1/v (min/nM)
0.600
0.400
0.200
0.000
-0.010
-0.006
-0.002
0.002
0.006
0.010
1/S (1/nM)

INTERNATIONAL SEARCH REI IT

International Application No PCT/EP 91/01811

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 9/00 C 12 N 15/00 A 61 K 31/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	Trends in Biotechnology, volume 8, no. 7, July 1990, Elsevier Science Publishers Ltd, (Cambridge, GB) J.J. Rossi et al.: "RNA enzymes (ribozymes as antiviral therapeutic agents", pages 179-183, see the whole article ---	1,26
A	Trends in Biotechnology, volume 8 no. 7, July 1990, Elsevier Science Publishers Ltd (Cambridge, GB) M. Cotten: "The in vivo application of ribozymes", pages 174-178, see the whole article ---	1,26
A	EP,A,0321201 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 21 June 1989, see the whole document, especially page 5 --- -/-	1,26

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25-11-1991	14. 01. 92
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: Reinke Steinberg

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9101811 SA 51441

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 20/12/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0321201	21-06-89	AU-A- 2800789	19-07-89
		WO-A- 8905852	29-06-89
WO-A- 8804300	16-06-88	US-A- 4987071	22-01-91
		EP-A- 0291533	23-11-88
		JP-T- 1501445	25-05-89
		US-A- 5037746	06-08-91
EP-A- 0387775	19-09-90	AU-A- 5130190	01-11-90
		CA-A- 2012312	16-09-90
		JP-A- 3130080	03-06-91

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No. 649074

Patents Act 1990

STANDARD PATENT

I, Bruce Ian Murray, Commissioner of Patents, grant a Standard Patent with the following particulars:

Name and Address of Patentee:
Max-Planck-Gesellschaft Zur Forderung Der Wissenschaften E.V., Bunsenstrasse 10 D-3400 Gottingen Federal Republic Of Germany

Names of Actual Inventors: Fritz Eckstein; Olaf Heidenreich; Wolfgang A Pieken; Fritz Benseler; David B Olsen and David M. Williams

Title of Invention: Modified ribozymes

Application Number: 86139/91

Term of Letters Patent: Sixteen years commencing on 23 September 1991

Priority Details:

Number	*Date*	*Country*
EP9001731	12 October 1990	WORLD INTELLECTUAL PROPERTY ORGANIZATION (WIPO)

Dated this 30 day of August 1994

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IHR ZEICHEN / YOUR REF.	UNSER ZEICHEN / OUR REF.	DATUM / DATE
GI 1298 195/036	AUSL 12297/HBbj	September 27, 1994

Patentanmeldung Nr. 86139/91 in Australien
Modified Ribozymes

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Patent 1

Sehr geehrte Damen und Herren,

die Patentanmeldung Nr. 86139/91 in Australien ist zur Erteilung gekommen. Anliegend übersenden wir Ihnen die Patenturkunde mit der Bitte um **Empfangsbestätigung.**

Bitte beachten Sie die nachstehenden Daten:

Patent Nr.: **649074**

Beanspruchte Priorität: October 12, 1990 EP PCT/EP90/01731

Beginn der Laufzeit: **September 23, 1991**

Längste Dauer: **16 ab dem 23.September 1991**

Fälligkeit der Jahresgebühren: jährlich am **23. September**

Wir werden Sie an die Fälligkeit der Jahresgebührenzahlungen rechtzeitig - jedoch ohne Haftung - erinnern.

Das Patent wurde am 30.August 1994 erteilt.

Mit freundlichen Grüßen

P.S. Original Patent
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Anlagen:
Patenturkunde

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NUCLEOZYMES

Field of the Invention

This invention relates to nucleozymes, mixed nucleic acid polymers having catalytic activity. The invention also relates to methods of preparing and using nucleozymes.

Background of the Invention

Proteins were the only known catalysts of cellular reactions until the discovery of RNA catalysts (ribozymes). In some instances, the folded structure of a ribozyme catalyses a cleavage reaction on another part of the same molecule (cis-reaction). In other instances (trans-reaction), the ribozyme may also act as a catalyst on another RNA or DNA molecule (substrate) by cleaving or ligating pieces of the substrate without changing itself in the process. (Zaug et al. Science, Vol. 231, pp. 470-75, 1986; Cech Science, Vol. 236, pp. 1532-39, 1987).

A well-characterized example of a ribozyme is the self-splicing Group I intron from the nuclear rRNA of Tetrahymena thermophila. An intron is an intervening sequence in a eukaryotic gene which does not encode a protein or in rare cases encodes a different protein. Introns are transcribed along with coding sequences (exons) to produce precursor RNA. The introns are removed from the precursor RNA and the exons are ligated by RNA cleaving and splicing steps. The Group I intron or ribozyme of T. thermophila catalyzes its own removal from the precursor RNA molecule. (Kruger et al. Cell

31:147-157, (1982); Zaug et al. (1986)). The self-splicing ribozyme catalyzes a variety of phosphodiester transfer reactions. The ribozyme can act as a ribonuclease, ligase, phosphotransferase, acid phosphatase, polymerase and RNA restriction endonuclease (Zaug, A.J., et al., Science 231:470-475 (1986); Zaug, A.J., et al., Nature 324:429-433 (1986); Zaug, A.J., et al., Biochemistry 25:4478-4482 (1986); Been, M.D., et al., Science 239:1412-1416 (1988); Doudna et al., Nature 339:519-522 (1989); all incorporated by reference herein).

The "hammerhead" and "hairpin" ribozymes also have been studied and described (Perreault et al., Nature 344:565-567 (1990); Perreault et al. Biochemistry 30:4020-25 (1991); Yang et al. Biochemistry 29:11156-60 (1990); Chowrira et al. Biochemistry 30:8518-22 (1991); Uhlenbeck Nature, 328:596-600 (1987)). The hammerhead ribozyme forms a stem loop secondary structure to form the catalytically active molecule. The hairpin ribozyme has a structure resembling a hairpin.

Although ribozymes are intriguing molecules, their use for in vivo applications is limited if not precluded. The all-RNA molecules are susceptible to degradation from enzymes (RNAses) present in vivo. There presently is no way known to inventors for delivering such molecules to the intended site in an active form.

Summary of the Invention

The present invention is based on the discovery that ribozymes have catalytically critical sites and that it is not necessary to have an all-RNA molecule to have catalytic activity. The ribozyme like molecules of the invention or "nucleozymes" have ribonucleotides or nucleic acid analogues (hereinafter NAAs) at catalytically critical sites and NAAs or deoxyribonucleotides at non-catalytically critical sites. The preferred nucleozymes have ribonucleotides at catalytically critical sites. Nucleozymes have catalytic activity on the same substrates as their ribozyme counterparts.

The nucleozymes of the present invention thus essentially are modified ribozymes having at least a portion, or all, of the ribonucleotides replaced with deoxyribonucleotides or NAAs. The nucleozymes are significantly more resistant to degradation than their all-RNA ribozyme counterparts because the chemicals or enzymes present in vivo do not recognize the nucleic acid internucleotide bonds. The resistance can be to either enzymatic or chemical degradation. Preferably, a majority of the ribonucleotides of the ribozyme are replaced with deoxyribonucleotides or NAAs. The stability of the nucleozymes allows them to be useful as therapeutic agents whereas ribozymes would be cleaved and rendered inactive by enzymes, e.g. RNAses, present in vivo.

The nucleozymes of the present invention are chimeric nucleic acid polymers having catalytic activity due to and preferably optimized by the presence of RNA or a NAA at a catalytically critical site. The present invention provides chemistry which allows synthesis of the chimeric polymers and the determination of catalytically critical sites. The sites may be determined by varying the location of deoxyribonucleotides in a chimeric polymer and determining the locations responsible for or related to the chimeric polymer's ability to catalyze.

The present invention also pertains to a method for making a chimeric polymer. The polymers are made by phosphitylating protected ribonucleotides or NAAs units under conditions to form substantially pure-protected phosphoramidites or synthons of a single isomer. The protected phosphoramidites are coupled to each other forming a protected chimeric nucleic acid chain. The protecting groups are removed from the chimeric nucleic acid chain under conditions which completely deprotect the polymer.

The method of the present invention alleviates problems associated with a known prior art method for preparing chimeric RNA/DNA polymers (Perreault et al. Nature 344:565-567 (1990); Wu et al. Journal of the American Chemical Society 111:8531-33 (1989)). The prior art synthetic method for making chimeric polymers had problems with the migration of the protecting groups during the phosphitylating step, difficulty in removing the protecting groups and also

has problems resulting from the process of removing the protecting groups in the deprotection step. The former problem results in the production of monomer units having protecting groups in an undesired position. The latter problem results, in many cases, in a) nucleotide modification, b) phosphodiester linkage isomerization, and c) to retention of a substantial amount of protecting groups on the polymer resulting in a non-functional polymer. The first problem was overcome in the present invention by selecting a catalyst capable of minimizing migration of protecting groups, e.g., a combination of 2,4,6-collidine and N-methylimidazole. The problem in removing the protecting groups was eliminated by deprotecting the mixed polymer in the presence of ethanolic ammonia.

The present invention also pertains to methods of using the nucleozymes. The nucleozymes may be used to perform the same catalytic functions as their all-RNA ribozyme counterparts. For example, a nucleozyme may be used as a ribonuclease, ligase, phosphotransferase, acid phosphatase, polymerase, or an RNA restriction endonuclease. The nucleozymes may be used to selectively cleave and ligate substrates by contacting the substrates with a nucleozyme such that the nucleozyme targets a specific sequence in the substrate for cleavage or ligation. The nucleozymes may be used as polymerases to polymerize the production of an oligoribonucleotide or an oligodeoxyribonucleotide. The nucleozymes also may be used in place of antisense RNA technology.

The nucleozymes also may be used as therapeutic agents introduced in vivo due to their resistance to chemical and enzymatic degradation. The nucleozymes may be used, for example, in a method for treating a subject for a retrovirus associated disease, e.g., human immunodeficiency virus (HIV). The method involves administering a therapeutically effective amount of at least one nucleozyme to the subject such that the nucleozyme cleaves the RNA genome of the retrovirus rendering it inactive. A plurality of nucleozymes also may be administered if it is desirable to target more than one sequence in the RNA genome.

A nucleozyme may be provided in a pharmaceutical composition. The pharmaceutical composition would include at least one nucleozyme and a pharmaceutically acceptable carrier.

It is an object of the present invention to provide a nucleozyme capable of maintaining its catalytic properties in vivo.

It is an object of the present invention to provide a chimeric nucleic acid polymer having catalytic activity.

It is yet another object of the present invention to provide a method for preparing chimeric polymers which are free of protecting groups and undesired isomeric side products.

It is yet another object of the present invention to provide a homogenous chimeric polymer.

Brief Description of the Drawings

FIG 1 depicts a structure of a hammerhead nucleozyme.

FIG 2 is a table listing nucleozymes of the present invention indicating the positions of ribonucleotides in the nucleozymes. The position designations correspond to the structure depicted in FIG 1.

FIGs 3 and 4 are photographs of a gel demonstrating the cleavage of a 5'-^{32}P-labeled RNA substrate. The designation "RNA" is the all-RNA containing ribozyme and the nucleozymes are as abbreviated in FIG 2.

FIG 5 is a photograph of a gel demonstrating the cleavage of a radioactive RNA substrate by nucleozymes of the present invention containing methoxy substituted NAAs. The nucleozymes are abbreviated as in FIG 2.

FIGs 6 and 7 are graphs depicting the stability of nucleozymes compared to the stability of the all-RNA ribozyme counterpart after exposure to both RNAse A (6) and a yeast cellular extract (7).

Detailed Description

The nucleozymes of the present invention have catalytic activity. Nucleozymes essentially are modified ribozymes preferably having at least one ribonucleotide or nucleic acid analogue (NAA) at a catalytically critical site(s) and deoxyribonucleotides or NAAs at non-critical sites.

The term "nucleozyme" is intended to include catalytic chimeric polymeric chains containing ribonucleotides and deoxyribonucleotides and/or nucleic acid analogues.

A ribozyme is an all-RNA containing molecule capable of being a biological catalyst. Ribozymes are art recognized and examples of such molecules include the "hammerhead" or "hairpin" ribozymes.

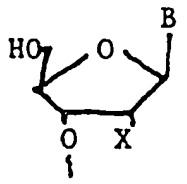
The term "catalytic activity" is intended to include many types of catalysis. For example, the catalytic activity could be that of a ribonuclease, ligase, phosphotransferase acid, phosphatase, polymerase, and RNA restriction endonuclease.

The term "nucleic acid analogue" (NAA) is intended to include analogues which are structurally similar to ribonucleotides or deoxyribonucleotides and are capable of being monomer units in a polymer capable of hybridizing with DNA or RNA. The analogue may impart properties to a polymeric chain which differ from those of a nucleotide but the analogue is capable of being a monomer unit in a polymeric chain. The NAA may impart resistance to chemical or enzymatic degradation to the chimeric polymer. NAAs may be selected for their structural conformation if a particular conformation is desired for the polymer. A NAA which is structurally similar to a ribonucleotide may be positioned at a catalytically site if the NAA is capable of participating and/or attaining the desired catalytic activity. Preferably, if the NAA is positioned at a

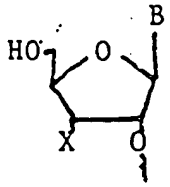
catalytically critical site it has a formula as depicted in Formulas I–IV below wherein X is a good coordinating ligand with divalent metal ions, eg., Mg^{+2}.

A nucleotide analogue may contain a heterocyclic ring as depicted in the formulae I–IV below or may be acyclic as shown in Formula V below. The preferred heterocyclic ring containing analogues of the present invention have three hydroxy substituents, alkoxy substituents or combinations thereof.

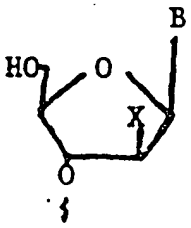
Formula (I)



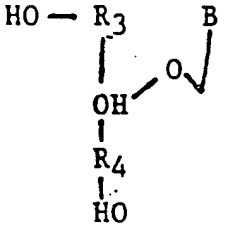
Formula (III)



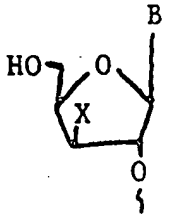
Formula (II)



Formula (V)



Formula (IV)



In the above formulae, Б is a base. The base may be substituted or unsubstituted. Examples of bases include adenine, cytosine, guanine, uracil,

2-aminopurine, hypoxanthine, imidazole, $R_1-\overset{\overset{O}{\|}}{C}-O-$,

R_1-SH-, and R_1-NH_2-. The term "base" is art-recognized and one of ordinary skill in the art would know which bases are useful in the present invention. X is selected from the group consisting of $-OR_1$, F, $-R_2OH$, $-NH_2$, $-R_2NH_2$, -Br, $-R_2Br$ and $-R_2F$ wherein R_1 is a lower alkyl group and R_2-R_4 are a lower alkyl hydrocarbon chains. The term "lower alkyl" is intended to include from one to six carbons, more preferably one to three carbons and most preferably a single carbon atom. For example, the most preferred X is methoxy.

B may be protected during the synthesis process. The protecting groups may be the conventional groups typically used in oligonucleotide synthesis processes e.g., N^6-benzoyl for adenine, N^4-benzoyl for cytosine, N^2-isobutyryl for guanine, and N^2-benzoyl for 2-aminopurine. Other useful protecting groups include phenoxyacetyl (PAC) and t-butoxyacetyl (TAC). One of ordinary skill in the art would know which protecting groups are appropriate for a particular base.

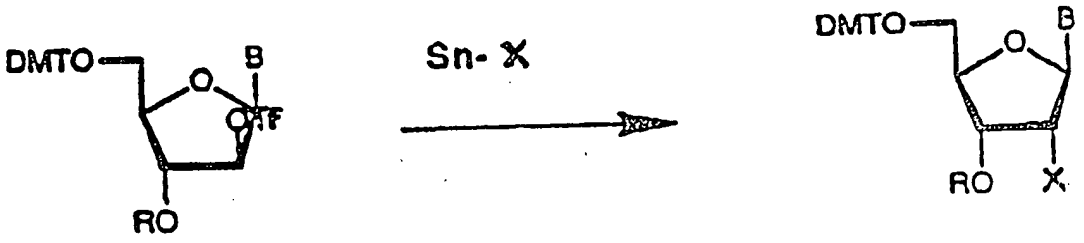
The NAAs capable of being positioned at a catalytically critical site may be determined by one of ordinary skill in the art using the following screening process. The catalytically critical sites of a particular ribozyme may be determined by positioning deoxyribonucleotides(s) at various locations within the ribozyme and evaluating the chimeric polymer's catalytic activity. After determining the locations of the catalytically

critical sites, NAAs may be substituted for the deoxyribonucleotide(s) and the chimeric polymer's catalytic activity is again evaluated. If the NAA containing chimeric polymer possesses catalytic activity then the NAA is suitable for positioning at a catalytically critical site.

The term "synthon" is intended to include the fully protected monomer units (phosphoramidites) used to assemble the nucleic acid analogues of a chimeric polymer chain. The term "nucleic acid analogue" is used to describe the units when polymerized as part of a chimeric polymer chain.

The term "chimeric polymer" is intended to include polymers containing at least two different types of monomer units, e.g., RNA, DNA, or NAA. For example, a chimeric polymer may include RNA/DNA, RNA/NAA, or DNA/NAA polymeric chains. It should be understood that the linkages between the building units of the polymeric chain may be linkages capable of bridging the units together for either in vitro or in vivo. For example, the linkage may be a phosphorous containing linkage, e.g., phosphodiester or phosphothioate, or may be a nitrogen containing linkage, e.g., amide. It should further be understood that the chimeric polymer may contain non-nucleotide spacer molecules along with its other nucleotide or analogue units. Examples of spacer molecules which may be used are described in Nielsen et al. Science, 254:1497-1500 (1991), the contents of which are expressly incorporated by reference.

The analogues described above may be prepared using synthetic methods capable of attaching the desired moieties at the selected positions. Other possible analogues will be apparent to one of ordinary skill in the art. Deoxyribonucleotides are readily available and may be purchased from a variety of sources. The alkyl substituted heterocyclic ring containing analogues may be synthesized by first reacting the moiety being modified with arabino triflates (trifluoromethane sulfonates) and second displacing the arabino triflates with an appropriate organotin compound selected on the basis of the desired moiety. A schematic of this reaction is depicted below:



This synthesis scheme may be used to prepare analogues wherein X is selected from the group consisting of $-OR_1$, $-R_2OH$, R_2F, -RBr, and $-RNH_2$. One of ordinary skill in the art would know how to synthesis the halogenated analogues (X is -F or -Br) and the amino substituted analogues (X is

$-NH_2$). These analogues may be synthesized as described by Williams et al., Biochemistry, 30:4001-4009 (1991), Doerr et al., J. Org. Chem., 32:1462 (1967), Mengel et al. Angew. Chem., 90:557 (1978), or Coddington et al., J. Org. Chem., 29:558 (1964), the contents of each of the references are hereby expressly incorporated by reference.

The acyclic nucleic acid analogues may be prepared by reacting a protected acyclic molecule with a diol. Reaction processes which may be used are described in Durand et al., Nucleic Acid Research, 18:6353 (1990); Seela et al., Nucleic Acid Research, 15:3113-3124 (1987); Cload et al., JACF, 113:6324-6326 (1991), the contents of each reference is hereby expressly incorporated by reference.

The nucleozymes have catalytically critical site(s) at which a ribonucleotide or NAA is necessary for the nucleozyme to have the desired level of catalytic activity. The term "catalytically critical site" is intended to include sites which, if altered from a ribonucleotide or a NAA to a deoxyribonucleotide, substantially reduces or even eliminates catalytic activity. A substantial reduction in catalytic activity would be that reduction which limits the usefulness of the nucleozyme as a catalyst in vitro or in vivo. The catalytically critical sites may be determined for each nucleozyme. Catalytically critical sites can be determined by preparing a variety of chimeric polymers using the chemical techniques described

herein and comparing the catalytic activity of the chimeric test polymers. The catalytically critical sites in the all-RNA containing ribozyme counterparts are determined by selecting sites believed to be involved in catalysis and inserting a deoxyribonucleotide at the particular site. If the chimeric polymer does not have the same or substantially the same catalytic activity as the all-RNA counterpart, then the selected site is presumed to be a catalytically critical site. The hammerhead nucleozyme has four catalytically critical sites which are the G9, G12, A13 and A29 positions for the sequence depicted in FIG 1.

The nucleozymes of the present invention are of a size capable of being synthesized using the chemistry described herein. Preferably, the nucleozymes have less than about 100 total building units, more preferably, less than about 80 building units, even more preferably, less than about 70 building units, and most preferably less than about 50 building units. Some nucleozymes may even have less than about 20 building units. The preferred nucleozyme is modeled after the hammerhead ribozyme, the catalytic portion of which has 35 building units. The term "building unit" is intended to include ribonucleotides, deoxyribonucleotides, or synthons.

The nucleozymes of the present invention are modified ribozymes having at least a portion of the ribonucleotides replaced with deoxyribonucleotides or

NAAs. The modified ribozymes or nucleozymes are significantly more resistant to degradation than the all-RNA counterparts. The degradation may be either enzymatic or chemical degradation. The language "significantly more resistant to degradation" is that resistance which allows the nucleozyme to remain largely intact for an extended period of time relative to its all-RNA counterpart. Preferably it has a resistance which allows it to be administered for in vivo applications.

Resistance to enzymatic degradation may be resistance to enzymes present in vivo, e.g. RNAses such as RNAse A. FIGs. 6 and 4B are graphs depicting the relationship between the percentage of the nucleozyme being intact at a particular RNAse A (FIG. 6) or yeast cellular extract (FIG. 7) concentration. The data set forth in FIGS. 6 and 7 was obtained using the following procedure. A sample of 0.001 pmol of 5' labeled nucleozyme or ribozyme was incubated with 0.5 mg of carrier TRNA in 50 mM Tris-HCl (pH 7.4) and 10 mM Mg^{+2} and different concentrations of RNAse A or yeast extract ten minutes for A, thirty minutes for extract. The reactions were stopped by the addition of 20 mM EDTA and loaded and analyzed on 15% PAGE in 7M urea. The yeast extract was prepared from a 1 ml culture of yeast strain BWG2-9A grown to late-lag phase, harvested and washed with 25 mM sodium phosphate buffer (pH 7.8). The pellet was suspended in 100 µl of the same buffer and sonicated for 20 seconds

(60W). After centrifugation for five minutes in an Eppendorf centrifuge, the supernatant was used directly after appropriate dilutions and incubations with the nucleozymes and the all-RNA ribozyme.

As shown in FIG. 6, the nucleozymes were at least about 75% intact at an RNAse A log concentration of -1.5 and at least about 80% intact at an RNAse A log concentration of -2.5. Preferably, the nucleozyme is at least about 90% intact at both concentrations. The difference in stability between the nucleozymes and ribozyme is even more apparent when the enzyme is a yeast cellular extract (FIG. 7). The nucleozymes were at least 90% intact at all of the tested concentrations.

The chemical degradation for purposes of this invention is intended to include resistance to chemicals present in vivo and in vitro. The resistance may be to alkaline hydrolysis, e.g., sodium hydroxide and water.

The preferred nucleozymes of the present invention are modified ribozymes having a majority of the ribonucleotides replaced with deoxyribonucleotides or NAAs. At least one of the units is a deoxyribonucleotide and preferably most of the units are deoxyribonucleotides. The nucleozymes more preferably, have at least about 75%, even more preferably at least about 85%, most preferably at least about 90% of their ribonucleotides replaced with deoxyribonucleotides or NAAs. The nucleozyme also may be made up entirely of NAAs or a combination of NAAs and deoxyribonucleotides.

The present invention also pertains to a method of making a chimeric polymer. The method is similar to that described by Scaringe et al. to produce an all-RNA polymer. (Nucleics Acid Research Vol. 18, No. 18, 5433-41 (1990)), the contents of which is expressly incorporated by reference. The method includes the steps of phosphitylating protected RNA, DNA or NAA units under conditions which minimize migration of the protecting group forming isomerically pure protected phosphoramidites. The protected RNA or synthons may have the desired moieties protected with the protecting groups capable of surviving the phosphitylation and coupling steps. Examples of such groups include conventional DNA protecting groups such as 5'-O-DMT, N-Bz (Ade and Cyt), N-iBu (Gua), ß-cyanoethyl for phosphate, TB DMS for 2'-hydroxyl.

The term "isomerically pure protected phosphoramidites" is intended to include phosphoramidite preparations free of a substantial amount of undesired isomers of the phosphoramidites. A substantial amount is that amount which would substantially interfere or impede with the preparation's ability to be used in forming a mixed polymeric chain. For example, a consideration in the chemical synthesis of a ribonucleotide phosphoramidite is contamination of the desired 2'-O-protecting group-3'-O phosphoramidite with the undesired 3'-O- protecting group -2'-O-phosphoramidite. Syntheses performed with the

latter lead to oligonucleotides having 5'-2' linkages. Isomerically pure protected phosphoramidites of the present invention lead to oligonucleotides which are free of such undesired linkages.

The method of the present invention also involves coupling the protected phosphoramidites together forming a protected chimeric polymeric chain. The coupling can be done using well-known chemical techniques known to one of ordinary skill in the art. Preferably the coupling is done on an automated synthesizer.

After the chimeric polymeric chain is formed, the protecting groups now can be removed from the chain under conditions which completely deprotect the polymer. Also, nucleotide base modification and/or phosphodiester linkage isomerization of the chain now can be minimized during the deprotection step.

The prevention of migration of the protecting groups in the phosphitylation step can be accomplished by phosphitylating in the presence of a catalyst selected to minimize migration. An example of such a catalyst is the combination of 2, 4, 6-collidine and N-methylimidazole.

The invention permits removal of the protecting groups in a manner that is complete and that minimizes nucleotide base modification and/or phosphodiester linkage isomerization. Complete removal of protecting groups includes substantially complete removal where a polymeric chain may have a

small number of protecting groups still attached which do not effect the polymeric chain's intended catalytic function. The deprotection step is accomplished by deprotecting the polymer in the presence of an agent capable of minimizing such effects. An example of such an agent is ethanolic ammonia.

The method also allows the production of a homogeneous RNA/DNA polymer free of undesired isomeric products. The term "free" is intended to include substantially free wherein a small amount of protecting groups or undesired isomers are present as long as the amount does not interfere or impede the polymer's function. The minimization of nucleotide base modification is intended to include that modification which would effect a polymeric chain's intended catalytic function. Minimizing phosphodiester linkage isomerization, when used in connection with a molecule, means preventing that degree of isomerization which would, adversely affect the molecule's intended catalytic function. Minimizing phosphodiester linkage isomerization when used in connection with a preparation, means preventing that degree of isomerization which would substantially affect the preparation's abilities to be used for its intended catalytic functions.

The present invention also pertains to methods for using the nucleozymes. The nucleozymes may be used for any method in which a ribozyme presently may be used. For example, the nucleozyme may be used to

selectively cleave an RNA substrate or to ligate two pieces of RNA together. When cleaving a substrate, the RNA substrate is contacted with at least one nucleozyme which targets a specific sequence in the substrate for cleavage. A plurality of nucleozymes also may be used in a cleavage process.

The nucleozymes of the present invention also may be used to polymerize an oligonucleotide molecule. The oligonucleotide may contain ribonucleotides, deoxyribonucleotides, and analogues of deoxy- or ribonucleotides. A template is contacted with a population of the appropriate nucleotide monomer units and a nucleozyme under conditions which allow an oligonucleotide complementary to the template to form. The template preferably is attached to a support.

The nucleozymes of the present invention also may be used for therapeutic methods due to their stability in vivo. The nucleozymes may be used, for example, to treat a subject for a retrovirus associated disease. A therapeutically effective amount of at least one nucleozyme is administered to a subject such that the nucleozyme(s) cleaves the RNA genome of the retrovirus or the viral mRNA rendering it inactive.

A retrovirus associated disease is intended to include diseases involving retroviruses. Retroviruses have an RNA genome making them susceptible to cleavage by the at least one nucleozyme or the plurality of nucleozymes. An example of such a retrovirus associated disease is AIDS wherein the causative retrovirus is the human immunodeficiency virus (HIV).

The term "subject" is intended to include living organisms susceptible to retroviruses, e.g., mammals. Examples of subjects include humans, cats, and rats.

The language "therapeutically effective amount" is intended to include that amount capable of eliminating or significantly reducing the symptoms associated with retrovirus associated diseases. The amount may be determined on an individual basis and will be based, at least in part, on consideration of the severity of symptoms to be treated, the results sought and the size of the subject. Thus, a therapeutically effective amount may be determined by one of ordinary skill in the art employing such factors using no more than routine experimentation.

The nucleozymes of the present invention also may be used in conjunction with or in place of antisense RNA technology. That is, to control the expression of a gene by targeting an appropriate mRNA. A nucleozyme may be selected based on its ability to target a particular mRNA sequence and subsequently an effective amount of the nucleozyme may be administered to a subject. The effective amount would be that amount necessary to target the RNA and control expression of a selected gene.

The present invention also pertains to pharmaceutical compositions containing at least one nucleozyme and a pharmaceutically acceptable carrier. The language pharmaceutically acceptable carrier is intended to include carriers capable of being co-administered with the nucleozyme(s) while not adversely affecting the nucleozyme(s) catalytic

activity. The carrier may be solid or liquid or a gel. Examples of liquid carriers include water, an aqueous solution of a non-toxic salt, e.g., sterile physiological saline solutions, or aqueous solutions containing organic solvents, e.g., ethanol. Also suitable are emulsions, such as oil-in-water. Solid carriers may include nutritive carriers, e.g., sucrose or gelatin, or non-nutritive carriers, e.g., cellulose or talc.

The Preferred Embodiment

The preferred nucleozymes of the present invention are modeled after the "hammerhead" ribozyme. The moderate size of the catalytic hammerhead domains of RNA lends itself to chemical synthesis. As shown in FIG. 1, the conserved hammerhead domain of around fifty ribonucleotides found in naturally occurring RNA has been petitioned between a thirty-five unit catalytic fragment 10, the ribozyme, and a fourteen nucleotide substrate unit 12.

The all-DNA analog of the hammerhead domain is inactive in catalysis. It was determined that nucleozymes containing as few as four ribonucleotides out of a total of thirty five nucleotides have catalytic activity. Active hammerhead nucleozymes require the presence of ribonucleotides in particular at four positions, particular the G9, G12, A13, and A29 positions shown in FIG. 2.

FIG. 2 is a table showing the chimeric polymers prepared along with the positions of ribonucleotides within the polymers. Nucleozymes are abbreviated as follows: DR and MR designate nucleozymes composed of

predominantly deoxyribonucleotides and 2'-methoxynucleotides, respectively. The following number indicates the number of ribonucleotides and the final number refers to a particular combination of ribonucleotides.

The all DNA analog of the hammerhead domain is inactive in catalysis. Several RNA/DNA mixed polymers appear as set forth and designated in the table of FIG 2. As shown in FIGs 3 and 4, the mixed polymer having ribonucleotides at positions 9, 10, 12, 13, 28, 29 and 30 of the ribozyme showed good catalytic activity (FIG 3). The nucleozyme containing the fewest ribonucleotides, DR4R3 and DR4R2, showed at least some activity. In FIGs 3 and 4, S indicates the mobility of the intact substrate and P, the product. RNA is the all-RNA ribozyme and nucleozymes are abbreviated as in FIG 1. The presence or absence of the enzymatic fragment is indicated by + or -, respectively in FIG 3. Lane 1 is a control lacking the added catalytic fragment in FIG 3. Lane 1 in FIG 4 is the 4.5 hour incubation of the substrate with neither Mg^{+2} nor nucleozyme and lane 2 is an incubation control in the presence of Mg^{+2} but no added nucleozyme. The samples were analyzed on 15% PAGE in 7M urea.

FIG 3 is a photograph showing the cleavage of radioactive substrates by OMe-nucleozymes. The S indicates the mobility of the intact substrate and P, the product. The reactions were performed as in FIG 2 except that the incubations of the substrate were with the MR5R nucleozyme, for four hours and the (Mg^{+2}) was varied. Lane 1 contained no Mg^{+2},

lane 2-10 mM, lane 3-20 mM, lane 4-30 mM and lane 5-50 mM. The OMe-nucleozymes are nucleozymes having synthons which are methoxysubstituted in the 2' position. The nucleozymes designated MR contain all methoxysubstituted nucleozymes rather than deoxyribonucleotides.

The catalytic activity of the nucleozymes was further evaluated by determining parameters including K_M and K_{cat} for some of the kinetic reactions. In addition, the K_{Mg}, K_s and k_3 of nucleozyme reactions were obtained to evaluate how extensive deoxyribonucleotide substitution effects Mg^{+2} cofactor binding. A summary of these results is presented in Table 1 below.

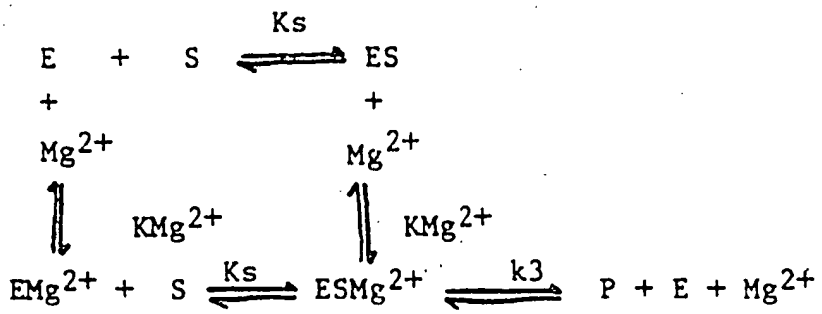
TABLE I Kinetic parameters of nucleozyme-catalyzed reactions[a]

Substrate/ Nucleozyme	K_M (μM)	Kcat (1/min)	K_S (μM)	K_3 (1/min)	K_{Mg} (mM)
RS/DR4R3	4.2	0.004	5	0.013	23
RS/DR5R2	4.9	0.013	4	0.040	24
RS/DR7R	4.5	0.067	5	0.21	24
RS/RR	0.7	1.2	0.8	1.8	8.1

[a] The kinetic experiments were performed under the standard conditions of FIG 2 with following differences: the final concentrations were: nucleozyme 0.05 μM; substrate from 0.5 μM to 5 μM; Mg^{2+} from 5 to 50 mM and the reaction times from 30 min to 6 h at 30°C. Initial rates were determined from the first 10% of the reactions. The chronology of substrate and metal ion addition is

currently not known. The values for Ks, k3 and KMg were, therefore, determined from the following random assembly model for ternary complex formation:

The generalized reaction scheme used to determine the K values is depicted below.



The reaction scheme starts with the catalytic species, the substrate and the Mg^{+2} cofactor, traversing a ternary complex and yielding a product. The apparent K_m's and K_s's of the three nucleozymes are virtually identical and approximately five times those of the all-RNA ribozyme acting on an RNA substrate.

The chimeric polymers were synthesized as follows:

Ribonucleotide, Deoxyribonucleotide, or 2'-Methoxy Analogue Phosphoramidite Synthesis and Purification

Dry N-Acyl-5'-O-DMT-2'-O-sily-ribonucleoside (10 mmol, 1 eq) was dissolved in 30 ml dry THF in a 300 ml round bottom flask. 2, 4, 6-Collidine (75 mmol, 7.5 eq) was added followed by N-methylimidazole (5 mmol, 0.5 eq). (N,N-dispropylamino) (cyanoethyl) phosphonamidic chloride (20 mmol, 2.0 eq [22 mmol,

2.2 eq in the case of guanosine or other nucleotide bases with reactive lactams]) was then added dropwise over 5 min at room temperature. The reaction was clear at the start but a white precipitate formed within 3 min (this is the collidine hydrochloride salt). The reaction was complete after 1-2 hour (determined by TLC). The reaction was then placed in an ice bath and diluted with 100 ml (1 volume) ethyl acetate. 150 ml 5% $NaHCO_3$ was added slowly (the first 10 ml over 5 minutes). (In the case of guanosine the reaction was first quenched with 5 ml of absolute ethanol). Heat was generated by the quenching of excess phosphitylating reagent. The mixture was then transferred to a separatory funnel and a second volume of ethyl acetate (100 ml) was added after first rinsing out the reaction flask. The aqueous phase was removed and the organic phase washed with saturated NaCl. The combined aqueous washes were back extracted with 50 ml. ethyl acetate and the combined organic phases were dried over Na_2SO_4. The solvent was removed in vacuo yielding a viscous oil. Coevaporation (x 5) with 50 ml toluene afforded the crude phosphoramidite as an offwhite foam or oil. Excess phosphinic acid and collidine caused it to be oily. After leaving the amidite under high vacuum overnight, resuspension and rotovaping of the amidite with methylene chloride and ethyl acetate usually produced a foam. The phosphoramidites were further purified by silica gel chromatography yielding a white foam in 75-85%

yields. This procedure was repeated for the preparation of the other desired phosphoramidites changing the initial protected nucleotide unit. The phosphoramidites were used to synthesize the mixed polymers using automated synthesis techniques commonly used for the synthesis of deoxyribonucleotides.

Automated Synthesis of Mixed Polymers

All syntheses were conducted on either a Gene Assembler Plus (Pharmacia), or a Cyclone (Milligen/Biosearch) synthesizer using standard protocols with an extended 12 min coupling step. A 30 fold excess (150 µl of 0.1 M = 15 mg, ~ 15 µmol) of the phosphoramidites and a 400 fold excess of tetrazole (400 µl of 0.5M = 200 µmol) relative to CPG bound 5'-hydroxyl was used in each coupling cycle. Synthesis scale was 0.5 µmol. Average coupling yields on the Gene Assembler Plus, monitored by an on-line colorimeter, were ~99.0% and on the Cyclone 97-98%, determined by colorimetric quantitation of the trityl fractions. Reaction columns for 0.5 µmol syntheses were Milligen/Biosearch 1.0 µmol columns. Oligonucleotide synthesis reagents: 1) for GA plus: detritylation solution was 2% TCA in ethylene dichloride; capping was performed with 20% N-Methyl imidazoyl in THF and 10% acetic anhydride/10% 2,6-lutidine in THF; oxidation solution was 0.02 MI_2, 1% lutidine, 10% water in tHF. Baker

Bio-Analyzed grade acetonitrile was further dried over activated 4 A molecular sieves. Tetrazole solution (0.5 M in acetonitrile was obtained from Applied Biosystems. 2) for Cyclone: all standard DNA synthesis ancillary reagents were used.

Deprotection of Mixed Polymers

The CPG-bound mixed polymer was transferred from the synthesis column to a 4 ml glass screw top vial. 1 ml of ethanolic ammonia was added and heated at 55°C for 16 hr. After cooling to -20°C, the ethanolic ammonia was removed from the CPG beads and the CPG was washed with 0.5 ml of 50:50/ethanol:water which was then added to the ethanolic ammonia. The combined supernatants containing the oligoribonucleotide were dried to a white powder. To remove the silyl protecting groups, the ammonia-deprotected mixed polymer was resuspended in 50 µl of 50:50/ethanol:water and 600 µl of 1M TBAF/THF and left at room temperature for about 24 hr. The solution was then added directly to 10 ml of 0.1M TEAB and loaded onto a Qiagen 500 anion exchange cartridge (Qiagen Inc., Studio City, CA) prewashed with 10 µl of 0.05M TEAB, the nucleozyme was eluted with 7 ml of 2M TEAB and dried down to a white powder.

Gel Purification of Fully Deprotected Mixed Polymers

The oligomers were first checked by analytical PAGE (0.75 mm x 20 cm x 45 cm). 1 ODU of oligonucleotide in 5 µl H_2O was added to 5 µl

of deionized formamide and the total 10 µl solution was loaded into a 1 cm wide lane. Following electrophoresis the gels were photographed by placing the gel over a fluorescent TLC plate and illuminating the gel with a UV lamp. The desired sequence was established according to electrophoretic mobility and purified by preparative electrophoresis using 1.5 mm thick gels with a single 12 cm wide lane. After electrophoresis the desired band was excised, crushed, placed into a sterile 5 ml test tube, and covered with 50 mM NH_4OAc pH 7.0. The tube was covered and kept at 37°C O/N. The supernatant was then removed and the gel pieces washed with an additional 1 ml of the extraction buffer. The combined washings were filtered through a 0.45 micron filter and loaded onto a 1 gram size Sep-Pak C18 cartridge (Waters-Millipore) prewashed with 5 ml each of acetonitrile, 50% acetonitrile/0.1M TEAB and 0.1M TEAB. After washing the cartridge with 5 ml of 0.1 M TEAB, the RNA was eluted in 5 ml 35:35:30 acetonitrile/methanol/water and dried down to a white powder.

The mixed polymers set forth in the Table of FIG 2 were prepared as described above.

EQUIVALENTS

Those skilled in the art will be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein.

These and all other equivalents are intended to be encompassed by the following claims.

What is claimed is:

CLAIMS

1. A nucleozyme having catalytic activity, comprising:

a ribonucleotide and at least one second unit being a deoxyribonucleotide or nucleic acid analogue, the nucleozyme having a ribonucleotide or nucleic acid analogue at a catalytically critical site.

2. The nucleozyme as claimed in claim 1 including at least one deoxyribonucleotide.

3. The nucleozyme as claimed in claim 2 wherein all of the second units are deoxyribonucleotides.

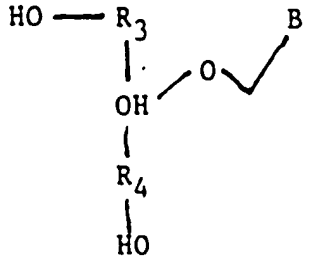
4. A nucleozyme as claimed in claim 1 wherein the second units include at least one nucleic acid analogue.

5. A nucleozyme as claimed in claim 1 wherein the second units include at least 50% nucleic acid analogues.

6. A nucleozyme as claimed in claim 4 wherein the analogue contains a heterocylic ring.

7. A nucleozyme as claimed in claim 4 wherein the analogue is acyclic.

8. A nucleozyme as claimed in claim 4 wherein the analogue has the following formula:



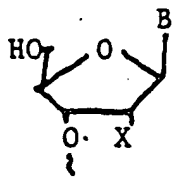
wherein B is a base and R_3 and R_4 are each independently lower alkyl groups.

9. A nucleozyme as claimed in claim 8 wherein B is selected from the group of adenine, cytosine, guanine, uracil, 2-aminopurine,

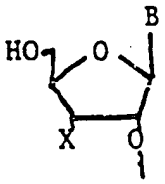
hypoxanthine, and imidazoyl, R_1C-O-, R_1-SH-, R_1-NH_2- wherein R_1 is a lower alkyl group.

10. A nucleozyme as claimed in claim 4 wherein the ribonucleotide analogue has a formula selected from the group consisting of:

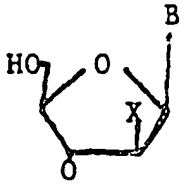
Formula (I)



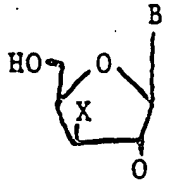
Formula (III)



Formula (II)



Formula (IV)



wherein B is a base and X is a good coordinating ligand for a divalent metal ion.

11. A nucleozyme as claimed in claim 10 wherein B is selected from the group consisting of adenine, cytosine, guanine, uracil, 2-aminopurine, hypoxanthine, and imidazoyl;

and X is selected from the group consisting of $-OR_1$, F, $-R_2OH$, and $-CH_2F$, wherein R_1 is a lower alkyl group and R_2 is a lower alkyl chain.

12. A nucleozyme as claimed in claim 10 wherein the analogue has a formula as shown in (I) and X is methoxy.

13. A nucleozyme as claimed in claim 1 wherein the nucleozyme has a ribonucleotide at a plurality of catalytically critical sites.

14. A nucleozyme as claimed in claim 1 wherein the nucleozyme has less than about 100 total building units.

15. A nucleozyme as claimed in claim 14 wherein the nucleozyme has less than about 80 total building units.

16. A nucleozyme as claimed in claim 15 wherein the nucleozyme has less than about 70 total building units.

17. A nucleozyme as claimed in claim 16 wherein the nucleozyme has less than about 50 total building units.

18. A nucleozyme as claimed in claim 1 wherein the nucleozyme has a sequence corresponding to the ribozyme selected from the group consisting of hammerhead and hairpin.

19. A nucleozyme as claimed in claim 18 wherein the ribozyme is the hammerhead ribozyme.

20. A nucleozyme as claimed in claim 19 wherein the catalytically critical sites are the G9, G12, A13, and A29 positions.

21. A nucleozyme as claimed in claim 1 wherein the nucleozyme is selected from the group consisting of DR4R2, DR4R3, DR5R2, DR5R3, DR6R2, DR7R, MR5R2, MR3R, and MR2R.

22. A nucleozyme having catalytic activity, comprising:

a modified ribozyme having at least a portion of its ribonucleotides replaced with deoxyribonucleotides or nucleic acid analogs, the modified ribozyme being significantly more resistant to degradation than its all-RNA counterpart.

23. A nucleozyme as claimed in claim 22 wherein the resistance is to enzymatic degradation.

24. A nucleozyme as claimed in claim 22 wherein the resistance is to chemical degradation.

25. A nucleozyme as claimed in claim 22 or 23 wherein the modified ribozyme is a modified hammerhead ribozyme.

26. A nucleozyme as claimed in claim 22 wherein the resistance is to enzymatic degradation and the nucleozyme is at least 80% intact at an RNase A log concentration of -2.5.

27. A nucleozyme as claimed in claim 26 wherein the nucleozyme is at least 90% intact.

28. A nucleozyme as claimed in claim 22 wherein the resistance is to enzymatic degradation and the nucleozyme is at least 75% intact at an RNase A log concentration of -1.5.

29. A nucleozyme as claimed in claim 28 wherein the nucleozyme is at least 90% intact.

30. A nucleozyme as claimed in claim 25 wherein the resistance is to enzymatic degradation and the nucleozyme is at least 80% intact at an RNase A log concentration of -2.5.

31. A nucleozyme as claimed in claim 30 wherein the nucleozyme is at least 90% intact.

32. A nucleozyme as claimed in claim 25 wherein the resistance is to enzymatic degradation and the nucleozyme is at least 75% intact and an RNase A log concentration of -1.5.

33. A nucleozyme as claimed in claim 32 wherein the nucleozyme is at least 90% intact.

34. A nucleozyme having catalytic activity comprising

a chimeric polymer made up of RNA and second units being deoxyribonucleotides or nucleic acid analogues, said chimeric polymer having catalytic activity and having a majority of its total units being deoxyribonucleotides or nucleic acid analogs.

35. A nucleozyme as claimed in claim 34 wherein at least one of the second units is a deoxyribonucleotide.

36. A nucleozyme as claimed in claim 34 wherein all of the second units are deoxyribonucleotides.

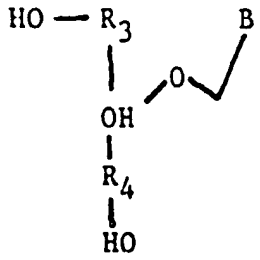
37. A nucleozyme as claimed in claim 34 wherein the second units include a nucleic acid analogue.

38. A nucleozyme as claimed in claim 37 wherein the second units include a plurality of nucleic acid analogues.

39. A nucleozyme as claimed in claim 37 wherein at least one of the nucleic acid analogues contains a heterocyclic ring.

40. A nucleozyme as claimed in claim 37 wherein the nucleic acid analogue is acyclic.

41. A nucleozyme as claimed in claim 38 wherein the analogue has the following formula:

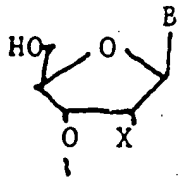


wherein B is a base and R_3 and R_4 are each independently lower alkyl groups.

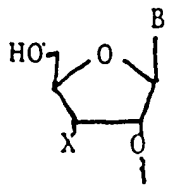
42. A nucleozyme as claimed in claim 39 wherein the base is selected from the group of adenine, cytosine, guanine, uracil, 2-aminopurine, hypoxanthine, and imidazoyl $R_1C(=O)-O-$, R_1-SH-, R_1-NH_2- wherein R_1 is a lower alkyl group.

43. A nucleozyme as claimed in claim 37 wherein the analogue has a formula selected from the group consisting of:

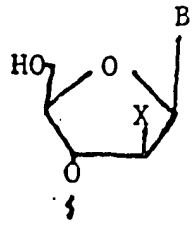
Formula (I)



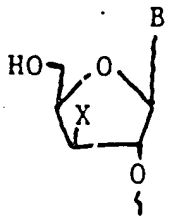
Formula (III)



Formula (II)



Formula (IV)



wherein B is a base and X is a good coordinating ligand with a divalent metal ion.

44. A nucleozyme as claimed in claim 40 wherein the base is selected from the group consisting of adenine, cytosine, guanine, uracil, 2-aminopurine, hypoxanthine, and imidazoyl;

and X is selected from the group consisting of $-OR_1$, F, $-R_2OH$, and $-CH_2F$, wherein R_1 is a lower alkyl group and R_2 is a lower alkyl chain.

45. A nucleozyme as claimed in claim 43 wherein the analogue has a formula as shown in (I) and X is methoxy.

46. A nucleozyme as claimed in claim 34 wherein all of the second units are 2'-methoxy ribonucleotide analogues.

47. A nucleozyme as claimed in claim 34 wherein the nucleozyme is a modified hammerhead ribozyme.

48. A nucleozyme as claimed in claim 34 or 47 wherein at least 50% of the polymer is second units.

49. A nucleozyme as claimed in claim 48 wherein at least 75% of the polymer is second units.

50. A nucleozyme as claimed in claim 49 wherein at least 85% of the polymer is second units.

51. A nucleozyme as claimed in claim 50 wherein at least 90% of the polymer is second units.

52. A chimeric polymer including at least one ribonucleotide and at least one second unit being a deoxyribonucleotide or nucleic acid analogue, the polymer having catalytic activity optimized by the presence of RNA at a catalytically critical site.

53. A chimeric polymer as claimed in claim 52 wherein the second units include a deoxyribonucleotide.

54. A chimeric polymer as claimed in claim 52 wherein RNA is present at all of the catalytically critical sites.

55. A chimeric polymer as claimed in claim 53 wherein all of the second units are deoxyribonucleotides.

56. A method of making a chimeric polymer comprising:

phosphitylating protected RNA or second units under conditions to form substantially pure-protected phosphoramidites of a single isomer, the second units being deoxyribonucleotides or nucleic acid analogues;

coupling the protected phosphoramidites together forming a protected chimeric polymer chain; and

deprotecting the chimeric polymer chain under conditions which completely deprotect the chimeric polymer.

57. A method as claimed in claim 56 wherein the second units in the phosphitylating step include a deoxyribonucleotide.

58. The method as claimed in claim 56 or 57 wherein the phosphitylating agent in the phosphitylating step is (N,M-disopropyl amino) (cyanoethyl) phosphonamidic chloride.

59. A method as claimed in claim 56 or 57 wherein the phosphitylating step is conducted in the presence of 2,4,6-collidine and N-methylimidazole.

60. A method as claimed in claim 58 wherein the phosphitylating step is conducted in the presence of 2,4,6-collidine and N-methylimidazole.

61. A method as claimed in claim 56 wherein the deprotecting step is conducted in the presence of ethanolic ammonia.

62. A method as claimed in claim 59 wherein the deprotecting step is conducted in the presence of ethanolic ammonia.

63. A chimeric polymer containing ribonucleotide units and second units joined together forming a chimeric polymer, the second units being at least one deoxyribonucleotide or nucleic acid analogue and the chimeric polymer being free of protecting groups.

64. A chimeric polymer as claimed in claim 63 wherein the second units include deoxyribonucleotides.

65. A homogeneous RNA/DNA polymer containing both deoxyribonucleotide and ribonucleotide units joined together forming a mixed polymer, the mixed polymer being free of undesired isomeric products.

66. A homogeneous polymer as claimed in claim 65 wherein the undesired isomeric products contain 5-2 linkages.

67. In a chimeric nucleotide polymer synthesis, the improvement comprising, the use of a catalyst capable of minimizing migration of protecting groups during a phosphitylation step.

68. A method as claimed in claim 67 wherein the catalyst is a combination of 2,4,6-collidine and N-methylimidazole.

69. In a chimeric nucleotide polymer synthesis, the improvement comprising, the removal of protecting groups from a protected mixed polymer in the presence of ethanolic ammonia.

70. A method for selectively cleaving an RNA substrate, comprising:

contacting a substrate with a nucleozyme which targets a specific sequence in the substrate for cleavage.

71. A method for treating a subject for a retrovirus associated disease, comprising:

administering a therapeutically effective amount of a nucleozyme to the subject such that the nucleozyme cleaves the RNA genome of the retrovirus or viral m RNA rendering it inactive.

72. A method as claimed in claim 71 wherein a plurality of nucleozymes are administered.

73. A method as claimed in claim 71 wherein the retrovirus is human immunodeficiency virus.

74. A pharmaceutical composition, comprising:
at least one nucleozyme; and
a pharmaceutically acceptable carrier.

75. A method of polymerizing an oligonucleotide molecule comprising:
contacting a template with a population of nucleotide monomer units and a nucleozyme such that an oligonucleotide complementary to the template is formed.

76. A method as claimed in claim 75 wherein the oligonucleotide formed is an oligoribonucleotide.

77. A method as claimed in claim 75 wherein the oligonucleotide formed is an oligodeoxyribonucleotide.

78. A method as claimed in claim 70 wherein the RNA is messenger RNA.

79. A method for controlling the expression of a gene in a subject comprising:
selecting a nucleozyme which targets an RNA sequence involved in the expression of a gene;
administering an effective amount of the selected nucleozyme to the subject such that the nucleozyme targets the RNA sequence and inhibits the expression of a gene.

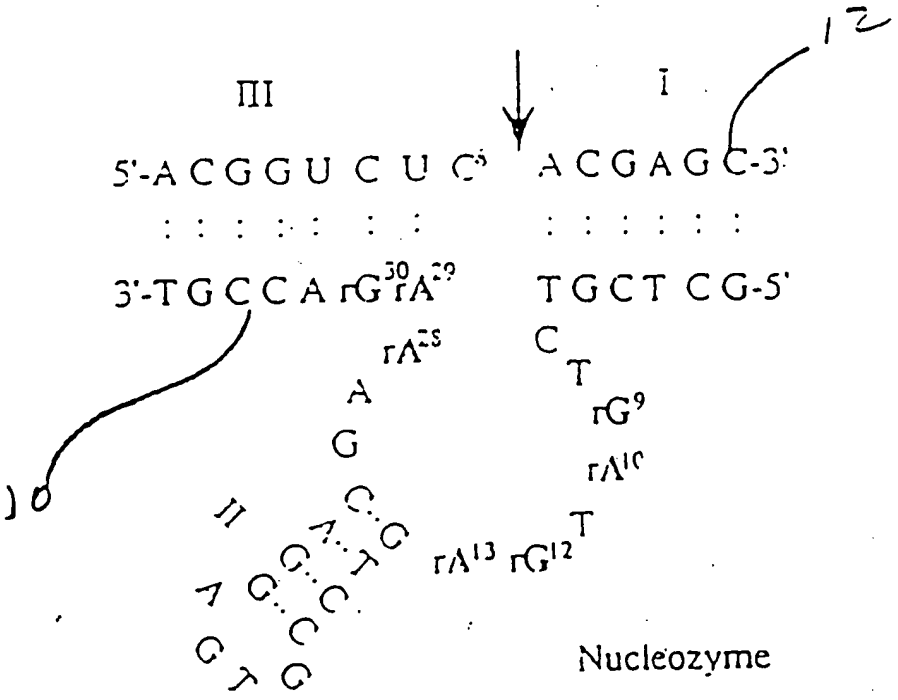


FIG 1

	Position of ribonucleotide		
	rGrA	rGrA	rArArG
	9 10	12 13	28 29 30
Nucleozyme			
DR2R	rG	rA	- -
DR3R	rG	rA	rA
DR4R1	rG	rA	rArG
DR4R2	rG	rGrA	rG
DR4R3	rG	rGrA	rA
DR5R2	rG	rGrA	rArG
DR5R3	rGrA	rGrA	rG
DR6R2	rGrA	rGrA	rArG
DR7R	rGrA	rGrA	rArArG
OMe-nucleozyme			
MR2R	rG	rA	- -
MR3R	rG	rA	rA
MR5R	rG	rGrA	rArG

FIG 2

FIG 3

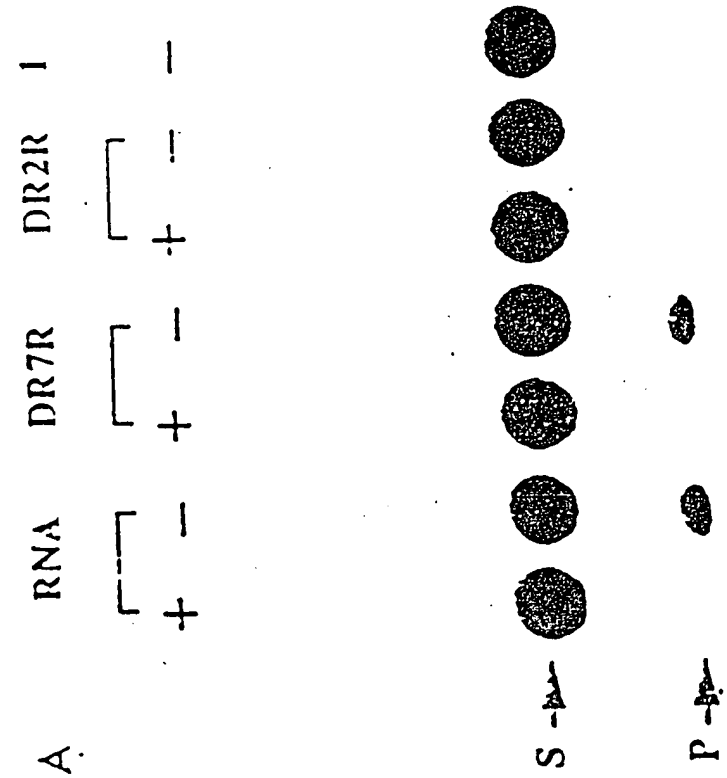


FIG 4

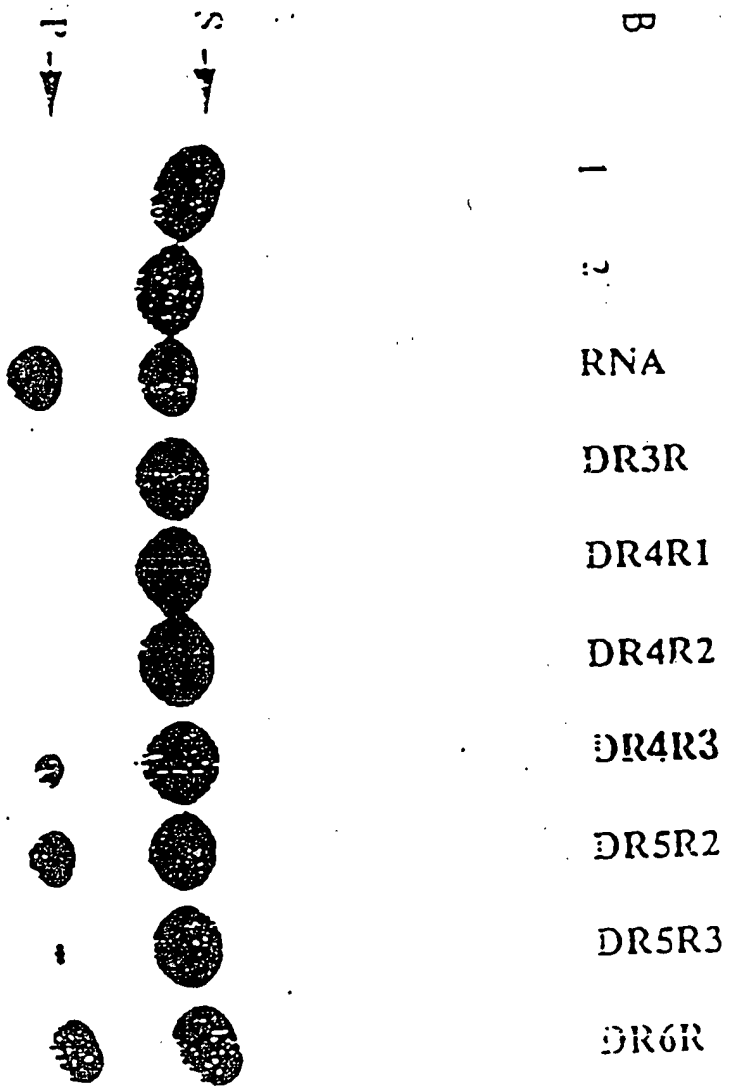


FIG 5

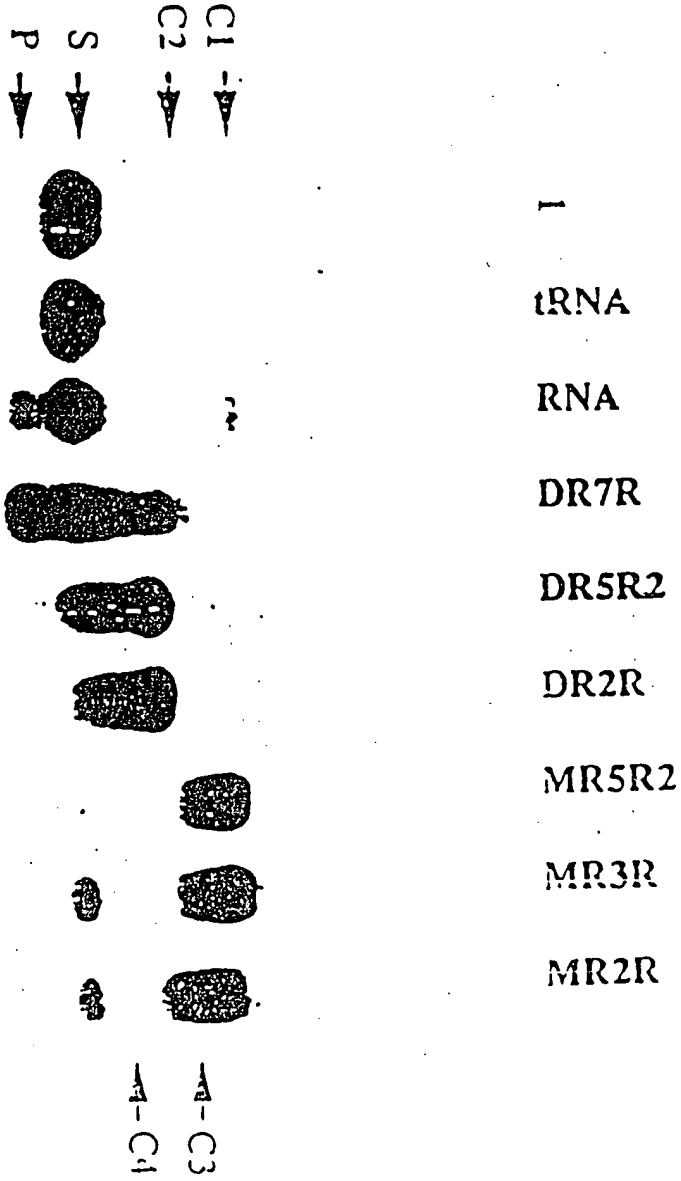


FIG 6

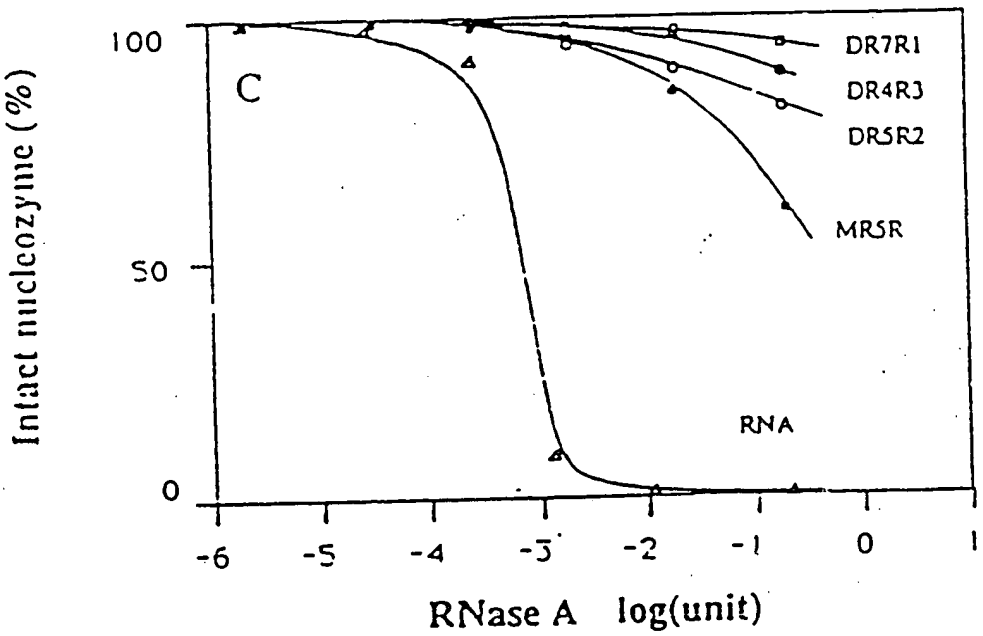
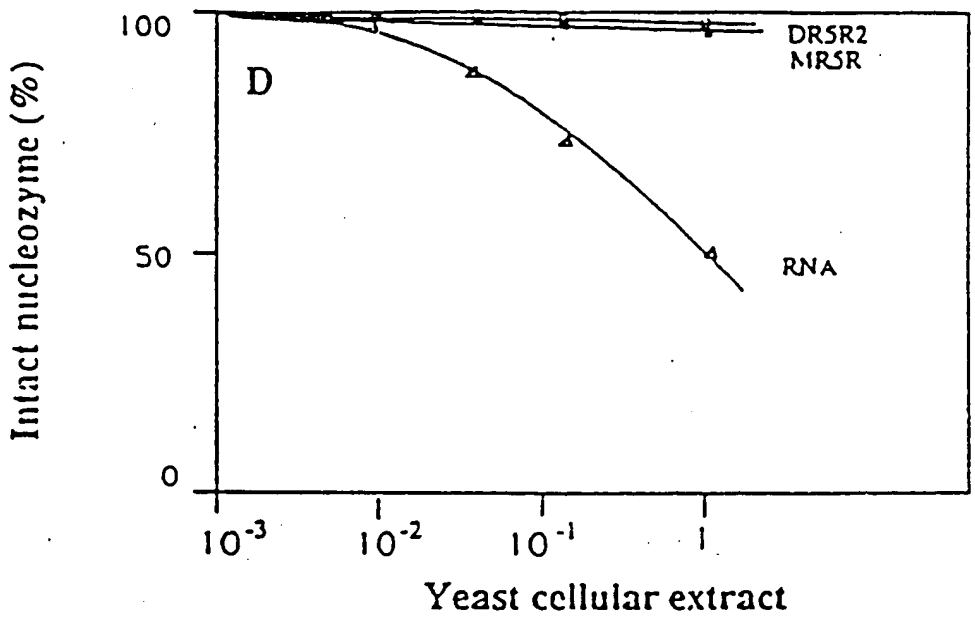

100
50
0
C
DR7R1
DR4R3
DR5R2
MR5R
RNA
-6
-5
-4
-3
-2
-1
0
1
Intact nucleozyme (%)
RNase A log(unit)

FIG 7


100
50
0
D
DR5R2
MR5R
RNA
Intact nucleozyme (%)
10^{-3}
10^{-2}
10^{-1}
1
Yeast cellular extract

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/00833

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N9/00; A61K31/70; C07H21/00; C12P19/34

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K ; C07H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 103 162 (CITY OF HOPE) 21 March 1991 see the whole document	1-3, 13-20 22-23, 25-36, 47-57, 63-66, 70-71 73-74, 78-79
Y		56-62, 67-69

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 MAY 1993	03. 06. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	NUCLEIC ACIDS RESEARCH vol. 18, no. 18, 25 September 1990, ARLINGTON, VIRGINIA US pages 5433 - 5441 SCARINGE, S.A. ET AL. 'Chemical synthesis of biologically active oligoribonucleotides using beta-cyanoethyl prtected ribonucleoside phosphoramidites' cited in the application	56-62
Y	see the whole document	56-62, 67-69

X	SCIENCE vol. 253, 19 July 1991, LANCASTER, PA US pages 314 - 317 PIEKEN, W.A. ET AL. 'Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes' see the whole document	1,4-6, 10-11, 13-20, 22-23, 25-34, 37-39 42-43, 47-48, 52,54, 56,63,70

X	BIOCHEMISTRY vol. 30, no. 16, 1991, EASTON, PA US pages 4020 - 4025 PERREAULT, J.-P. ET AL. 'Relationship between 2'-hydroxyls and magnesium binding in the hammerhead RNA domain: a model for ribozyme catalysis' cited in the application see the whole document	1-3, 13-20, 34-35, 47, 52-54, 63-66,70

X	WO,A,8 804 300 (UNIVERSITY PATENTS, INC.) 16 June 1988 see page 16, line 10 - page 17, line 24; claim 72	75-76

X	SCIENCE vol. 251, 20 March 1991, LANCASTER, PA US pages 1605 - 1608 DOUDNA, J.A. ET AL. 'A multisubunit ribozyme that is a catalyst of and template for complementary strand RNA synthesis' see the whole document	75-76

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	BIOCHEMISTRY vol. 31, no. 21, 2 June 1992, EASTON, PA US pages 5005 - 5009 YANG, J.-H. ET AL. 'Minimum ribonucleotide requirement for catalysis by the RNA hammerhead domain' see the whole document	1,4-6, 10-34, 37-39, 42-52, 54,56, 58-63 67-70
P,X	EMBO JOURNAL vol. 11, no. 5, May 1992, EYNSHAM, OXFORD GB pages 1913 - 1919 PAOLELLA, G. ET AL. 'Nuclease resistant ribozymes with high catalytic activity' see the whole document	1,4-6, 10-20, 22-34, 37-39, 42-43, 45-52,63 70
P,X	WO,A,9 207 065 (MAX-PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 30 April 1992 see page 4, line 6 - page 7, line 3 see claims; example 4	1,4-6, 10-11 13-20 22-34, 37-39, 42-44 47-52, 54,63, 70-71, 73-79